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(54) Title: PEPTIDES WHICH ENHANCE TRANSPORT OF AN ACTIVE AGENT ACROSS TISSUES AND COMPOSITIONS AND METHODS OF USING THE SAME

(57) Abstract

Targeting agents that are capable of permitting or facilitating transport of an active agent through a human or animal gastro-intestinal tissue. These targeting agents are peptides or their derivatives (e.g., fragments) and peptidomimetics thereof, and the nucleotide sequences coding for the peptides and derivatives. The targeting agents have use in facilitating transport of active agents from the lumenal side of the GIT into the systemic blood system, and/or in targeting active agents to the GIT. Therapeutic methods of administration, pharmaceutical compositions and formulations based on the targeting agent peptides are also provided. Preferably, the active agent is a drug or drug-containing nano- or microparticle.

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Peptides Which Enhance Transport of an Active Agent Across Tissues and Compositions and Methods of Using the Same

Technical Field

The present invention relates to peptides which permit or facilitate the transport of drugs, macromolecules, or particles, such as drug-loaded nanoparticles, through human or animal tissues. In particular, this invention relates to certain targeting peptides, their derivatives or peptidomimetics, and pharmaceutical compositions containing the same that are designed to target one or more transport or uptake pathways. These targeting agents enhance the delivery and/or transport of an active agent through tissue, such as epithelial cells lining the lumenal side of the gastro-intestinal tract (GIT) when administered to a subject, especially when administered orally.

Background Art

The epithelial cells lining the lumenal side of the GIT are a major barrier to drug delivery following oral administration. However, there are four recognized transport pathways which can be exploited to facilitate drug delivery and transport: the transcellular, paracellular, carrier-mediated and transcytotic transport pathways. The ability of a conventional drug, peptide, protein, macromolecule or nano- or microparticulate system to "interact" with one of these transport pathways may result in increased delivery of that drug or particle from the GIT to the underlying circulation.

In the case of the receptor-mediated, carrier-mediated or transcytotic transport pathways, some of the "uptake" signals have been identified. These signals include, *inter alia*, folic acid, which interacts with the folate receptor, mannose and cetylmannoside, which interact with the mannose receptor, and cobalamin, which interacts with Intrinsic Factor. In addition, leucine- and tyrosine-based peptide sorting motifs or internalization sequences exist, such as YSKV, FPHL, YRGV, YQTI,

TEQF, TEVM, TSAF, YTRF, which facilitate uptake or targeting of proteins from the plasma membrane to endosomes. Phage display libraries can be screened using specific membrane receptors or binding sites to identify peptides that bind specifically to the receptor or binding site. The ability of certain motifs or domains of peptides or proteins to interact with specific membrane receptors, followed by cellular uptake of the protein:receptor complex may point towards the potential application of such motifs in facilitating the delivery of drugs. However, the identification of peptides or peptide motifs by their ability to interact with specific receptor sites or carrier sites, such as sites expressed on the apical side of the epithelial sites of the GIT, may not be able to determine, or may not be the most effective way to determine, the identity of peptides capable of enhancing the transport of an active agent, especially a drug-loaded nano- or microparticle, through tissues such as epithelial lining.

Non-receptor-based assays to discover particular ligands have also been used. For instance, a strategy for identifying peptides that alter cellular function by scanning whole cells with phage display libraries is disclosed in Fong et al., *Drug Development Research* 33:64-70 (1994). However, because whole cells, rather than intact tissue or polarized cell cultures, are used for screening phage display libraries, this procedure does not provide information regarding sequences whose primary function includes affecting transport across polarized cell layers.

Additionally, Stevenson et al., *Pharmaceutical Res.* 12(9), S94 (1995) discloses the use of Caco-2 monolayers to screen a synthetic tripeptide combinatorial library for information relating to the permeability of di- and tri-peptides. While useful, this technique does not assess the ability of the disclosed di- and tri-peptides to enhance delivery of a drug, especially a drug-loaded nano-or microparticle formulation.

Co-pending PCT applications WO 97/17613 and WO 97/17614 disclose a method of identifying a peptide which permits or facilitates the transport of an active agent through a human or animal tissue. A predetermined amount of phage from a random phage library or preselected phage library is plated unto or brought into contact with a first side, preferably the apical side, of a tissue sample, either *in vitro, in vivo* or *in situ,* or polarized tissue cell culture. At a predetermined time, the phage

which is transported to a second side of the tissue opposite the first side, preferably the basolateral side, is harvested to select transported phages. The transported phages are amplified in a host and this cycle of events is repeated (using the transported phages produced in the most recent cycle) a predetermined number of times, such as from zero to six times, to obtain a selected phage library containing phage which can be transported from the first side to the second side. Lastly, the sequence of at least one random peptide coded by phage in the selected phage library is determined in order to identify a peptide which permits or facilitates the transport of an active agent through a human or animal tissue.

Dynamin, a 100 kDa member of the GTPase superfamily, is required for endocytosis at the plasma membrane in a large number of cell types, including neurones. Dynamin was first identified as a brain protein localized in nerve terminals in association with clathrin-coated vesicles and a specific role of dynamin in synaptic vesicle recycling was proposed. The recent studies of dynamin mutants in non-neuronal cells suggest a general role in receptor-mediated endocytosis. Dynamin concentrates on clathrin-coated membrane surfaces and spontaneously self-assembles into helical arrays forming rings and stacks of interconnected rings at the necks of invaginated coated pits that become constricted. It appears that a concerted conformational change then closes the rings and pinches off the budding coated vesicles from the plasma membrane.

In mammals, Dynamin is encoded by at least three different genes: dynamin I, which is predominantly expressed in neurons where it is concentrated in synaptic terminals; dynamin II, which has a widespread tissue distribution and dynamin III, which is expressed primarily in the testis but also in lung and brain. In rats, dynamin I and III expression has been shown to be upregulated throughout development of the brain whereas the levels of dynamin II mRNA remain unchanged. The three dynamin proteins encoded by these genes have a similar molecular weight of 94-100kDa, are approximately 70% identical to each other, and have a similar domain structure.

Common structural and functional domains can be identified in all three Dynamin forms: a GTP-binding domain in the amino-terminal region (300 amino acids with three consensus elements highly conserved among all GTPase superfamily

members), a central pleckstrin homology (PH) domain, a highly basic proline-rich carboxyl terminus, and a stretch of amino acids with a propensity to form coiled-coil structures positioned between the PH domain and the proline-rich domain. The PH domain is a stretch of approximately 100 amino acids at the center of the dynamin molecule and is generally thought to be involved in protein-protein or protein-lipid interaction.

The C-terminal tail of Dynamin (~100 amino acids) is characterized by a very high pI and is rich in proline residues (therefore termed PRD). Proline is a unique amino acid due to its cyclic structure, which in polypeptide chains restricts the natural conformation of surrounding amino acids. PRDs are therefore characteristically stiff structures that may protrude out from the protein to form sticky arms able to bind to a range of proteins. The dynamin PRD region contains at least three domains which can interact with proteins containing src-homology (SH3) domains (for example, in amphiphysin, Grb2, isoforms of phospholipase C (PLC), PI3 kinase, among others). Such interaction is of great interest as SH3-containing proteins are involved in signal transduction pathways mediating protein-protein and protein-cytoskeleton interactions. It has also been proposed that specific regions within the PRD mediate specific targeting of dynamin to clathrin-coated pits, indicating that the key targeting signal for dynamin is an SH3 protein.

The highly basic nature of the C-terminal domain of dynamin also enables its interaction with acidic phospholipids, phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, endogenous brain vesicles and may, in part, explain the association of dynamin with plasma membranes. These ionic interactions seem to stimulate assembly of dynamin dimers, trimers, etc. and, as consequence of self-assembly, GTP hydrolysis is activated. The PRD region can be phosphorylated by PKC *in vitro* and this phosphorylation stimulates intrinsic dynamin GTPase activity.

The common routes of therapeutic drug administration are oral ingestion or parenteral (intravenous, subcutaneous and intramuscular) routes of administration. Intravenous drug administration suffers from numerous limitations, including (i) the risk of adverse effects resulting from rapid accumulation of high concentrations of drug, (ii) repeated injections which can cause patient discomfort; and (iii) the risk of

infection at the site of repeated injections. Subcutaneous injection is not generally suitable for delivering large volumes or irritating substances. Whereas oral administration is generally more convenient, it is limited where the therapeutic agent is not efficiently absorbed by the gastrointestinal tract. To date, the development of oral formulations for the effective delivery of peptides, proteins and macromolecules has been an elusive target. Poor membrane permeability, enzymatic instability, large molecular size, and hydrophilic properties are four factors that have remained major hurdles for peptide and protein formulators (reviewed by Fix, J.A., 1996, J. Pharmac. Sci. 85:1282-1285). In order to develop an efficacious oral formulation, the peptide must be protected from the enzymatic environment of the gastrointestinal tract (GIT), presented to the absorptive epithelial barrier in a sufficient concentration to effect transcellular flux (Fix, J.A., 1996, J. Pharmac. Sci. 85:1282-1285), and if possible "smuggled" across the epithelial barrier in an apical to basolateral direction.

Site specific drug delivery or drug targeting can be achieved at different levels, including (i) primary targeting to a specific organ, (ii) secondary targeting to a specific cell type within that organ and (iii) tertiary targeting where the drug is delivered to specific intracellular structures (*e.g.*, the nucleus for genes) (reviewed in Davis and Ilum, 1994, In: Targeting of Drugs 4, (Eds), Gregoriadis, McCormack and Poste, 183-194). At present there is a considerable amount of ongoing research work in the Drug Delivery Systems (DDS) area, and much of it addresses (i) targeting delivery and (ii) the development of non-invasive ways of getting macromolecules, peptides, proteins, products of the Biotechnology industry, etc. into the body (Evers, P., 1995, Developments in Drug Delivery: Technology and Markets, Financial Times Management Report). It is generally accepted that targeted drug delivery is crucial to the improved treatment of certain diseases, especially cancer, and not surprisingly many of the approaches to targeted drug delivery are focused in the cancer area. Many anticancer drugs are toxic to the body as well as to malignant cells. If a drug, or a delivery system, can be modified so that it "homes in" on the tumor, then by maximizing the drug concentration at the disease site, the anti-cancer effect can be exploited to the full, while toxicity is greatly reduced. Tumors contain antigens which provoke the body to respond by producing antibodies designed to attach to the

antigens and destroy them. Monoclonal antibodies are being used as both delivery vehicles targeted to tumor cells (reviewed by Pietersz, G.A., 1990, Bioconjugate Chem. 1:89-95) and as imaging agents to carry molecules of drug or imaging agent to the tumor surface.

There exists a need for targeting agents and delivery systems that incorporate the targeting agents that are capable of enhancing the transport and/or uptake of an active agent though one or more tissue barriers. There is a particular need for peptide targeting agents, derivatives or peptidomimetics thereof that are effective in transporting drugs, including drug-loaded nano- and microparticles delivery systems, across a human or animal tissue barrier. Because of the advantages associated with oral administration of an active agent to a patient, including safety and patient-acceptability factors, there exists a particular need for targeting agents and delivery systems that can enhance the transport of an active agent across the epithelial layers in the GIT, especially with respect to poorly water-soluble active agents and low permeability active agents. A special need exists for agents capable of increasing the bioavailability following oral administration of an active agent having an intended site of action at a site distant from the GIT, such as the brain, lung, testis and the like.

Disclosure of the Invention

The present invention provides targeting agents that permit or facilitate transport of an active agent through a human or animal gastro-intestinal tissue. These targeting agents are peptides or their derivatives (*e.g.*, fragments) and peptidomimetics thereof, and the nucleotide sequences coding for the peptides and derivatives. These targeting agents can be used to target an active agent to one or more transport and/or uptake pathways, such as endocytosis, carrier mediated uptake, transcytosis and pinocytosis.

Preferably, the tissue through which transport and/or uptake is facilitated is of the duodenum, jejunum, ileum, ascending colon, transverse colon, descending colon, or pelvic colon. The tissue is most preferably epithelial cells lining the lumenal side of the GIT.

The targeting agents of the present invention have use in facilitating transport of active agents from the lumenal side of the GIT into the systemic blood system, and/or in targeting active agents to the GIT. Thus, for example, by binding (covalently or noncovalently) a peptide of the invention to an orally administered active agent, the active agent can be targeted to receptor sites or transport pathways which are known to operate in the human gastrointestinal tract, thus facilitating its absorption into the systemic system. Additionally, the targeting agents of this invention have use in facilitating targeted drug delivery across other barrier systems in which the respective target receptor or transport and/or uptake pathway is found, for example, the blood brain barrier, the vasculature and the lung. The targeting agents also have use in facilitating targeted drug delivery to tissues or cells in which the respective target receptor or uptake and/or transport pathway is found, including but not limited to the brain, heart, lung testis, spleen, liver, tumors, the eye and the like.

Additionally, the transport and/or uptake mediating targeting agents disclosed in this invention can be used in combination with other targeting agents which bind to or target specific receptors. Likewise, the transport and/or uptake mediating targeting agents disclosed in this invention can be used in combination with agents that permit or allow mucoadhesion. In this manner, effective delivery of an active agent can advantageously occur via different mechnisms simultaneously thus providing enhanced bioavailability of the active agent.

The targeting agents can be used to coat the surface of nanoparticulate or microparticulate drug delivery vehicles. Such coatings can be performed by either direct adsorption of the targeting agent to the surface of the particulate system or alternatively by covalent coupling of the targeting agent to the surface of the particulate system, either directly or via a linking moiety or by covalent coupling of the targeting agent to the polymers used in the production of nanoparticulate or microparticulate drug delivery vehicles, followed by the utilization of such targeting agent:polymer conjugates in the production of nanoparticulate or microparticulate drug delivery vehicles.

The invention also relates to derivatives and peptidomimetics of the peptides which are functionally active, *i.e.*, they are capable of displaying one or more known

functional activities associated with the full-length peptide such as the ability to bind or compete with the full-length peptide for binding to epithelial cells.

The invention further relates to fragments of (and derivatives thereof) of the peptides which comprise one or more motifs of the targeting agent peptide.

Antibodies to the targeting agent peptides and targeting agent peptide derivatives are additionally provided. Such antibodies are useful to quantify the targeting agents to the drug delivery system, to quantify the uptake of the targeting agent or delivery system across a tissue barrier such as the GIT, and to quantify targeted drug delivery to target cells or tissues.

Methods of production of the targeting agent peptides, derivatives, fragments and analogs, *e.g.*, by recombinant means, are also provided.

The present invention also relates to therapeutic methods, pharmaceutical compositions and formulations based on the targeting agent peptides. Formulations of the invention include but are not limited to targeting agent peptides or motifs and derivatives (including fragments) thereof; antibodies thereto; and nucleic acids encoding the targeting agent peptides or derivatives associated with an active agent. Preferably, the active agent is a drug or drug-containing nano- or microparticle.

Preferably, the active agent is a drug or a nano- or microparticle. More preferably, the active agent is a drug encapsulated or drug loaded nano- or microparticle, such as a biodegradable nano- or microparticle, in which the peptide is physically adsorbed or coated or covalently bonded, such as directly linked or linked via a linking moiety, onto the surface of the nano- or microparticle. Alternatively, the peptide can form the nano- or microparticle itself or can be directly conjugated to the active agent. Such conjugations include fusion proteins in which a DNA sequence coding for the peptide is fused in-frame to the gene or cDNA coding for a therapeutic peptide or protein, such that the modified gene codes for a recombinant fusion protein in which the "targeting" peptide is fused to the therapeutic peptide or protein and where the "targeting" peptide increases the absorption of the fusion protein from the GIT.

Additionally, viral vector systems can be modified to express the targeting agents on their surface such that the targeting agent is expressed as a fusion protein

with a viral surface protein and, as such, permit the targeting of the virus to receptor systems or transport and/or uptake pathways found on target cells or tissues. Such modified viral vector systems can be used for gene delivery.

Brief Description of the Drawings

Fig. 1 shows the binding profiles of brain selected phage to Caco-2 fixed cells. Fig. 1(A): Caco-2 P34, read at 30 min; Fig. 1(B): Caco-2 P36, read at 20 min; and Fig. 1(C); Caco-2 P34, read at 10 min. The neat sample corresponds to 10^{10} phage/well;

Fig. 2 shows the uptake and transport of brain selected phage 41.1 and 1.4 across Caco-2 cell monolayers. Fig. 2(A) shows the titer of phage eluted by acid treatment of cell monolayers after 2 hours incubation at 37°C; Fig. 2(B) shows the titer of cell-associated phage detected in the cell lysate after 2 hours incubation at 37°C; and Fig. 2(C) shows the titer of phage from the basolateral medium after 6 hours incubation at 37°C of Caco-2 cells with phage suspension on the apical side;

Fig. 3 shows the first ten homologies for each of the eight targeting peptides expressed by phage transported from the rat duodenal lumen to the brain obtained from a Blast search against the non-redundant SwissProt database.;

Figs. 4(A), 4(B) and 4(C) show selected homologies between peptides 41.1, 37.1 and 1.4 and known peptides/proteins, respectively;

Fig. 5 shows the evaluation of the synthetic peptides dynamin 41.2 and peptide 41.1 in ELISA assays for binding to membrane P100 (Fig. 5A) and cytosolic S100 (Fig. 5B) fractions isolated from Caco-2 cells. The samples consisted of the following dansylated peptides: (1) negative control scrambled PAX2-coated nanoparticles, (2) positive control HAX42-coated nanoparticles, (3) 41.1-coated

particles, and (4) 41.2-coated particles. The subcellular fractions P100 and S100 were used at a concentration of 50 μg/ml and the background has been subtracted;

Fig. 6 shows the binding of particles that are coated with dansylated targeting peptides and particles that are not coated to P100 (Fig. 6A) and S100 (Fig. 6B) Caco-2 fractions. The subcellular fractions P100 and S100 were used at a concentration of 10, 5, 1, and 0.1 μg/well whereas the coated particles were used at 62.5 μg/well. The background has not been subtracted. TEXP 939 refers to particles that are not coated with peptide; TEXP 1635 refers to particles that are coated with negative control scrambled PAX2; TEXP 2041 refers to particles that are coated with the brain selected targeting peptide 41.1; and TEXP 2042 refers to particles that are coated with dynamin human homologous 41.2; and

Fig. 7 shows the insulin plasma levels following intestinal administration of negative control (PBS); insulin particles (no peptide; 300 iu); positive control insulin HAX42-particles (40 mer; 300iu); and study group particles insulin peptide 41.1 particles (labeled Clone 41.1 particles; 43 mer; 300 iu) and insulin dynamin 41.2 particles (43 mer; 300 iu) according to this invention. The particles were administered in a rat duodenal open loop model.

Modes for Carrying out the Invention

This invention discloses specific targeting agents comprising targeting peptides and their derivatives and peptidomimetics thereof that are capable of facilitating the delivery or transport of an active agent such as a drug across human or animal tissues, including without limitation GIT epithelial layers, alveolar cells, endothelial cells of the blood-brain barrier, vascular smooth muscle cells, vascular endothelial cells, renal epithelial cells, M cells of the Peyers Patch, and hepatocytes. Furthermore, delivery systems, e.g., nanoparticles, microparticles, liposomes, micelles, and viral vector systems could be coated externally with, be linked to or be comprised of these "homing" peptides to permit targeted delivery of encapsulated drugs across particular tissues. In addition, fusion proteins can be synthesized, either

in vivo or *in vitro*, whereby the peptide is fused in-frame to a therapeutic peptide or protein active agent such that the peptide enhances the delivery or transport of the therapeutic peptide or protein across the tissue.

As used herein, the term human or animal "tissue" includes, without limitation, the duodenum, jejunum, ileum, ascending colon, transverse colon, descending colon, pelvic colon, the vascular endothelium which line the vascular system, the vascular endothelial cells which form the blood brain barrier, vascular smooth muscle, alveolar, liver, kidney, bone marrow, heart, spleen, pancreas, thymus, brain, spinal, neuronal and retinal eye tissue. As used herein, the phrase "human or animal tissue" refers to animal tissue explicitly including human tissue.

As used herein, the term "active agent" includes, without limitation, imaging agents or any drug or antigen or any drug- or antigen-loaded or drug- or antigen-encapsulated nanoparticle, microparticle, liposome, viral vector system or micellar formulation capable of eliciting a biological response in a human or animal. Examples of drug- or antigen-loaded or drug- or antigen-encapsulated formulations include those in which the active agent is encapsulated or loaded into nano- or microparticles, such as biodegradable nano- or microparticles, and which have the peptide adsorbed, coated or covalently bonded, such as directly linked or linked via a linking moiety, onto the surface of the nano- or microparticle. Additionally, the peptide can form the nano- or microparticle itself or the peptide can be covalently attached to the polymer or polymers used in the production of the biodegradable nano- or microparticles or drug-loaded or drug-encapsulated nano- or microparticles or the peptide can be directly conjugated to the active agent. Such conjugations to active agents include fusion proteins in which a DNA sequence coding for the peptide is fused in-frame to the gene or cDNA coding for a therapeutic peptide or protein such that the modified gene codes for a recombinant fusion protein.

Any drug known in the art may be used, depending upon the disease or disorder to be treated or prevented, and the type of subject to which it is to be administered. As used herein, the term "drug" includes, without limitation, any pharmaceutically active agent. Representative drugs include, but are not limited to, peptides or proteins, hormones, analgesics, anti-migraine agents, anti-coagulant

agents, anti-emetic agents, cardiovascular agents, anti-hypertensive agents, narcotic antagonists, chelating agents, anti-anginal agents, chemotherapy agents, sedatives, anti-neoplastics, prostaglandins, and antidiuretic agents. Typical drugs include peptides, proteins or hormones such as insulin, calcitonin, calcitonin gene regulating protein, atrial natriuretic protein, colony stimulating factor, betaseron, erythropoietin (EPO), interferons such as α, β or γ interferon, somatropin, somatotropin, somatostatin, insulin-like growth factor (somatomedins), luteinizing hormone releasing hormone (LHRH), tissue plasminogen activator (TPA), growth hormone releasing hormone (GHRH), oxytocin, estradiol, growth hormones, leuprolide acetate, factor VIII, interleukins such as interleukin-2, and analogs thereof; analgesics such as fentanyl, sufentanil, butorphanol, buprenorphine, levorphanol, morphine, hydromorphone, hydocodone, oxymorphone, methadone, lidocaine, bupivacaine, diclofenac, naproxen, paverin, and analogs thereof; anti-migraine agents such as heparin, hirudin, and analogs thereof; anti-coagulant agents such as scopolamine, ondansetron, domperidone, etoclopramide, and analogs thereof; cardiovascular agents, anti-hypertensive agents and vasodilators such as diltiazem, clonidine, nifedipine, verapamil, isosorbide-5-mononitrate, organic nitrates, agents used in treatment of heart disorders and analogs thereof; sedatives such as benzodiazeines, phenothiozines and analogs thereof; narcotic antagonists such as naltrexone, naloxone and analogs thereof; chelating agents such as deferoxamine and analogs thereof; anti-diuretic agents such as desmopressin, vasopressin and analogs thereof; anti-anginal agents such as nitroglycerine and analogs thereof; anti-neoplastics such as 5-fluorouracil, bleomycin and analogs thereof; prostaglandins and analogs thereof; and chemotherapy agents such as vincristine and analogs thereof. Representative drugs also include but are not limited to antisense oligonucleotides, genes, gene correcting hybrid oligonucleotides, ribozymes, aptameric oligonucleotides, triple-helix forming oligonucleotides, inhibitors of signal transduction pathways, tyrosine kinase inhibitors and DNA modifying agents. Drugs that can be used also include, without limitation, systems containing gene therapeutics, including viral systems for therapeutic gene delivery such as adenovirus, adeno-associated virus, retroviruses, herpes simplex virus, sindbus virus, liposomes, cationic lipids, dendrimers, and enzymes. For

instance, gene delivery viruses can be modified such that they express the targeting peptide on the surface so as to permit targeted gene delivery.

Targeting agent peptides, derivatives and peptidomimetics

The invention relates to targeting agent peptides and derivatives (including but not limited to fragments) and peptidomimetics thereof. In specific embodiments, of the present invention, such targeting agent peptides include but are not limited to those containing as primary amino acid sequences, all or part of the amino acid sequences substantially as depicted in Tables 4 and 7.

Derivatives of the targeting agent peptides include peptides in which certain amino acid residues are replace or substituted by other amino acid residues of similar properties. By way of example, an acidic residue could be replace by another acidic residue, a hydrophobic residue could be replace by another hydrophobic residue, a non-charged residue could be replaced by another non-charged residue. Additionally, any residue or residues of the targeting agent peptides can be replace by other natural or non-natural residues so as to either increase affinity or specificity for a target receptor or transport/uptake pathway or, alternatively, to broaden binding or interaction with other related receptors or transport pathways, to increase stability *in vivo* to protease digestion or other chemical or molecules detrimental to the targeting agent peptide.

In a specific embodiment of the invention, targeting agent peptides may contain multiple repeats comprising all or a fragment of a targeting agent peptide, in particular a fragment of the same comprising the targeting domain or that domain which facilitates drug absorption *in vivo* or *in vitro*.

In another specific embodiment of the invention, a combination of targeting agents are incorporated into the drug delivery system, preferably having at least one targeting agent component which targets a specific receptor site while the other targeting agent component targets an endogenous transport and/or uptake pathway in a target cell or tissue. By way of example, the active agent could be modified to target a receptor in the GIT together with one or more targeting agents identified in this invention, which target transport and/or uptake pathways.

Nucleic acids encoding such peptides, derivatives and peptide analogs are also provided. Furthermore, in view of the degeneracy of the genetic code, the nucleic acid sequence can be modified by substitution of one codon with another codon without changing the identity of the targeting agent peptide coded for by the resulting nucleic acid sequence or nucleic acid sequences. Related nucleic acid sequences are envisaged which code for derivatives of the targeting agent peptide, including those in which certain amino acid residues are replace or substituted by other amino acid residues of similar properties. By was of example, nucleic acid sequences are envisaged which code for targeting agent peptides in which an acidic residue could be replaced by another acidic residue, a hydrophobic residue could be replace by another hydrophobic residue, a non-charged residue could be replaced by another non-charged residue. In addition, nucleic acid sequences are envisaged which code for targeting agent peptides in which any residue or residues of the targeting agent peptides can be replaced by other natural residues so as to either increase affinity or specificity of the resulting targeting agent ligands for a target receptor or transport pathway, or alternatively, to broaden binding or interaction with other related receptors or transport pathway, to increase stability *in vivo* to protease digestion or other chemicals or molecules detrimental to the targeting agent peptide.

In a specific embodiment of the invention, nucleic acid sequences may code for proteins that contain multiple repeats comprising all or a fragment of a targeting agent peptide, in particular, a fragment or fragments of the same comprising the targeting domain or the domain that facilitates drug absorption *in vivo* or *in vitro*.

Targeting agent peptides whose amino acid sequence comprise, or alternatively, consists of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, and 18 or a portion thereof that facilitates the delivery and/or transport of an active agent across human or animal tissues are provided.

The production and use of derivatives and peptidomimetics related to the targeting agent peptides are within the scope of the present invention. In a specific embodiment, the derivative or peptidomimetic is functionally active, i.e., capable of exhibiting one or more functional activities associated with a full-length targeting agent peptides. As one example, such derivatives or peptidomimetics which have the

desired immunogenicity or antigenicity can be used, for example, in immunoassays, for immunization. In a preferred aspect, the derivatives or analogs have the ability to bind to Caco-2 cells, *in vitro*, or to intestinal tissue, *in vivo*, or to other non-epithelial cells or tissues *in vivo* or *in vitro*. In a specific embodiment of the invention, peptidomimetics may contain multiple repeats comprising all or a fragment of a targeting agent peptide or peptidomimetic, particularly a fragment comprising the targeting domain or domains which facilitate drug absorption *in vivo* or *in vitro*.

In particular, derivatives of the targeting agent peptide sequences can be made by substitutions, additions or deletions that provide for functionally equivalent molecules. Due to the degeneracy of nucleotide coding sequences, other nucleotide sequences which encode substantially the same amino acid sequence may be used in the practice of the present invention. These include but are not limited to nucleotide sequences which are altered by the substitution of different codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change. Likewise, the targeting agent peptide derivatives of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of a targeting agent peptide including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

In a specific embodiment of the invention, proteins consisting of or, alternatively, comprising all or a fragment of a targeting agent peptide consisting of at least 5, 10, 15, 20, 25, 30 or 35 (contiguous) amino acids of the full-length targeting

agent peptide are provided. In a specific embodiment, such peptides are not more than 20, 30, 40, 50, or 75 amino acids in length. In another specific embodiment of the invention, peptides may contain multiple repeats comprising all or a fragment of a targeting agent peptide, particularly a fragment comprising the targeting domain or that domain or domains which facilitate drug absorption *in vivo* or *in vitro*. Derivatives or analogs targeting agent peptides include but are not limited to those molecules comprising regions that are substantially homologous to targeting agent peptides or fragments thereof (*e.g.*, at least 50%, 60%, 70%, 80% or 90% identity) (*e.g.*, over an identical size sequence or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art) or whose encoding nucleic acid is capable of hybridizing to a coding targeting agent peptide sequence, under stringent, moderately stringent, or nonstringent conditions.

The targeting agent peptides, derivatives and peptidomimetics of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, the cloned targeting agent peptide gene sequence can be modified by any of numerous strategies known in the art (Maniatis, T., 1990, Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). The sequence can be cleaved at appropriate sites with restriction endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the production of the gene encoding a derivative of targeting agent peptides, care should be taken to ensure that the modified gene remains within the same translational reading frame uninterrupted by translational stop signals, in the gene region where the desired targeting agent peptides activity is encoded.

Additionally, the targeting agent peptides-encoding nucleic acid sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used, including but not limited to, chemical mutagenesis, *in vitro* site-directed mutagenesis

(Hutchinson, C., et al., 1978, J. Biol. Chem 253:6551), use of TAB® linkers (Pharmacia), use of PCR primers containing mutation(s) for use in amplification, etc.

Manipulations of the targeting agent peptide sequence may also be made at the protein level. Included within the scope of the invention are targeting agent peptide fragments or other derivatives or analogs which are differentially modified during or after translation or chemical synthesis, *e.g.*, by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, $NaBH_4$; acetylation, formylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; etc. In a specific embodiment, the amino- and/or carboxy-termini are modified.

In addition, targeting agent peptides and derivatives thereof can be chemically synthesized. For example, a peptide corresponding to all or a portion of a GIT transport receptor-binding peptide or multiple repeats of a domain or domains within the targeting agent peptide which comprises the desired domain or which mediates the desired activity *in vitro*, can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the GIT transport receptor-binding peptide sequence. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, α-amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, γ-Abu, ε-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β-methyl amino acids, Cα-methyl amino acids, Nα-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

In a specific embodiment, the targeting agent peptide derivative is a chimeric, or fusion, peptide comprising a targeting agent peptide or fragment thereof or multiple

repeats thereof (preferably consisting of at least a domain or motif of the targeting agent peptide, or at least 6, 10, 15, 20, 25, 30 or all amino acids of the targeting agent peptides or a binding portion thereof) joined at its amino- or carboxy-terminus via a peptide bond to an amino acid sequence of a different peptide. In one embodiment, such a chimeric peptide is produced by recombinant expression of a nucleic acid encoding the protein (comprising a transport receptor-coding sequence joined in-frame to a coding sequence for a different protein). Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, such a chimeric product may be made by protein synthetic techniques, *e.g.*, by use of a peptide synthesizer. Chimeric genes comprising portions of targeting agent nucleic acid fused to any heterologous protein-encoding sequences may be constructed. A specific embodiment relates to a chimeric protein comprising a fragment targeting agent peptides of at least six amino acids.

The targeting agent peptides and derivatives thereof of the invention can be assayed for binding activity by suitable *in vivo* or *in vitro* assays, *e.g.*, as described in the examples *infra* and/or as will be known to the skilled artisan.

Other specific embodiments of derivatives and analogs are described in the subsection below and examples sections *infra.*

In a specific embodiment, the invention relates to targeting agent peptide derivatives, in particular targeting agent peptide fragments and derivatives of such fragments, that comprise, or alternatively consist of, one or more domains of a targeting agent peptide. In particular, examples of such domains are identified in the examples *infra.*

The peptides and derivatives of the present invention may be chemically synthesized or synthesized using recombinant DNA techniques.

Alternatively, peptides may be prepared chemically by methods that are known in the art. For example, in brief, solid phase peptide synthesis consists of coupling the carboxyl group of the C-terminal amino acid to a resin and successively adding N-alpha protected amino acids. The protecting groups may be any known in

the art. Before each new amino acid is added to the growing chain, the protecting group of the previous amino acid added to the chain is removed. The coupling of amino acids to appropriate resins is described by Rivier et al., U.S. Patent No. 4,244,946. Such solid phase syntheses have been described, for example, by Merrifield, 1964, J. Am. Chem. Soc. 85:2149; Vale et al., 1981, Science 213:1394-1397; Marki et al., 1981, J. Am. Chem. Soc. 103:3178 and in U.S. Patent Nos. 4,305,872 and 4,316,891. In a preferred aspect, an automated peptide synthesizer is employed.

Purification of the synthesized peptides can be carried out by standard methods including chromatography (*e.g.*, ion exchange, affinity, and sizing column chromatography, high performance liquid chromatography (HPLC)), centrifugation, differential solubility, or by any other standard technique.

Biological peptide libraries can be used to express and identify peptides that bind to GIT transport receptors. According to this second approach, involving recombinant DNA techniques, peptides can by way of example be expressed in biological systems as either soluble fusion proteins or viral capsid proteins.

According to the invention, a targeting peptide, its fragments or other derivatives, or analogs thereof, may be used as an immunogen to generate antibodies which immunospecifically bind such an immunogen. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library.

Various procedures known in the art may be used for the production of polyclonal antibodies to a targeting peptide or derivative or analog. For the production of antibody, various host animals can be immunized by injection with the native targeting peptides, or a synthetic version, or derivative (*e.g.*, fragment) thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and

potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

For preparation of monoclonal antibodies directed toward a GIT transport receptor-binding peptides sequence or analog thereof, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology (PCT/US90/02545). According to the invention, human antibodies may be used and can be obtained by using human hybridomas (Cote et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030) or by transforming human B cells with EBV virus *in vitro* (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, pp. 77-96). In fact, according to the invention, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851-6855; Neuberger et al., 1984, Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing the genes from a mouse antibody molecule specific for the targeting peptides together with genes from a human antibody molecule of appropriate biological activity can be used; such antibodies are within the scope of this invention.

According to the invention, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce targeting peptide-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al., 1989, Science 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for GIT transport receptor-binding peptides, derivatives, or analogs.

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not

limited to: the $F(ab')_2$ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment, the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent, and Fv fragments.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, *e.g.* ELISA (enzyme-linked immunosorbent assay). For example, to select antibodies which recognize a specific domain of a targeting peptide, one may assay generated hybridomas for a product which binds to a GIT transport receptor-binding peptide fragment containing such a domain.

Antibodies specific to a domain of a targeting peptide are also provided.

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the targeting peptide sequences of the invention, *e.g.*, for imaging these peptides after *in vivo* administration (*e.g.*, to monitor treatment efficacy), measuring levels thereof in appropriate physiological samples, in diagnostic methods, etc. For instance, antibodies or antibody fragments specific to a domain of a targeting peptide or to a derivative of a peptide, such as a dansyl group or some other epitope introduced into the peptide, can be used to 1) identify the presence of the peptide on a nanoparticle or other substrate; 2) quantify the amount of peptide on the nanoparticle; 3)measure the level of the peptide in appropriate physiological samples; 4) perform immunohistology on tissue samples; 5) image the peptide after *in vivo* administration; 6) purify the peptide from a mixture using an immunoaffinity column or 7) bind or fix the peptide to the surface of a nanoparticle. This last use envisions attaching the antibody (or fragment of the antibody) to the surface of a drug-loaded nanoparticles or other substrate and then incubating this conjugate with the peptide. This procedure results in binding of the peptide in a certain fixed orientation, resulting in a particle that contains the peptide bound to the antibody in such a way that the peptide is fully active.

Compositions and Uses

The invention provides compositions comprising the targeting agent peptides, derivatives and peptidomimetic of the invention bound to a material comprising an active agent. Such compositions have use in targeting the active agent to the GIT and/or in facilitating transfer through the lumen of the GIT into the systemic circulation. Where the active agent is an imaging agent, such compositions can be administered *in vivo* to image the GIT (or particular transport receptors thereof).

In a preferred embodiment, a active agent is therapeutically or prophylactically administered to a human patient.

In a preferred embodiment, the invention provides for treatment of various diseases and disorders by administration of a therapeutic compound (termed herein "Therapeutic"). Such "Therapeutics" include but are not limited to: targeting peptides, and derivatives (including fragments) and peptidomimetics thereof that are capable of facilitating the delivery or transport of an active agent across human or animal tissues, bound to an active agent of value in the treatment or prevention of a disease or disorder (preferably a mammalian, most preferably human, disease or disorder). Therapeutics also include but are not limited to nucleic acids encoding the targeting agent peptides, derivatives or peptidomimetics bound to such a therapeutic or prophylactic active agent. The active agent is preferably a drug.

The invention provides methods of treatment (and prophylaxis) by administration to a subject of an effective amount of a Therapeutic of the invention. In a preferred aspect, the Therapeutic is substantially purified. The subject is preferably an animal, including but not limited to animals such as cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably human.

As will be clear, any disease or disorder of interest amenable to therapy or prophylaxis by providing a drug *in vivo* systemically or by targeting a drug *in vivo* to the GIT (by linkage to a targeting agent peptide, derivative or peptidomimetic of the invention) can be treated or prevented by administration of a Therapeutic of the invention. Such diseases may include but are not limited to hypertension, diabetes,

osteoporosis, hemophilia, anemia, cancer, migraine, and angina pectoris, to name but a few.

Any route of administration known in the art may be used, including but not limited to oral, nasal, topical, intravenous, intraperitoneal, intradermal, mucosal, intrathecal, intramuscular, etc. Preferably, administration is oral; in such an embodiment the targeting agent peptide, derivative or peptidomimetic of the invention acts advantageously to facilitate transport of the therapeutic active agent through the lumen of the GIT into the systemic circulation.

The present invention also provides therapeutic compositions or formulations. In a specific embodiment of the invention, a targeting agent peptide or motif of interest is associated with a therapeutically or prophylactically active agent, preferably a drug or drug-containing nano- or microparticle. More preferably, the active agent is a drug encapsulating or drug loaded nano- or microparticle, such as a biodegradable nano- or microparticle, in which the peptide is physically adsorbed or coated or covalently bonded, such as directly linked or linked via a linking moiety, onto the surface of the nano- or microparticle. Alternatively, the peptide can form the nano- or microparticle itself or can be directly conjugated to the active agent. Such conjugations include fusion proteins in which a DNA sequence coding for the peptide is fused in-frame to the gene or cDNA coding for a therapeutic peptide or protein, such that the modified gene codes for a recombinant fusion protein in which the "targeting" peptide is fused to the therapeutic peptide or protein and where the "targeting" peptide increases the absorption of the fusion protein from the GIT. Preferably the particles range in size from 200-600 nm.

Thus, in a specific embodiment, a targeting agent peptide is bound to a slow-release (controlled release) device containing a drug. In a specific embodiment, polymeric materials can be used (*see* Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, J. Macromol. Sci. Rev. Macromol. Chem. 23:61 (1983); *see also* Levy et al., Science 228:190 (1985); During et al., Ann. Neurol. 25:351 (1989); Howard et al., J. Neurosurg. 71:105 (1989)).

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a Therapeutic, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the Therapeutic, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient.

The Therapeutics of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium,

potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of the Therapeutic of the invention which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances.

Identification of targeting agent peptides

It has previously been shown that the NH_2-terminal amino acid sequence of the absorption proteins pIII and pVIII coded by *Escherichia coli* filementous bacteriophage phage such as fd, can be modified by recombinant DNA technology to include a library of random peptide sequences of defined length (Cwirla et al., *Proc. Natl. Acad. Sci. USA* 87:6378-6382 (1990)). Thus, a DNA library of modified phage fd sequences, coding for variable pIII or pVIII proteins can be constructed and propagated in *E. coli*.

Phage display libraries such as these can be used in a random screening approach or a preselected phage library or subpopulation from a phage display library in a preselected screening approach in order to determine the identity of peptide sequences which enhance the delivery of the bacteriophage from either the apical to basolateral side or the basolateral to apical side of either cultured model systems or in *in vitro, in situ* or *in vivo* tissue samples. Peptides that enhance the delivery from the apical to basolateral side (e.g., gut side to blood side) can be used to enhance the delivery of active agents in that direction. The converse holds for peptides that enhance the delivery from the basolateral to the apical side. For instance, plating on the basolateral side might determine peptides useful for raising a mucosal immune response to an antigen administered IV, subcutaneously, transdermally or by the opthalmic route.

The peptides coded by triple (or multiple) DNA inserts have the capacity to code for longer and/or more diverse peptides. Such longer peptides have a greater capacity to adopt secondary and tertiary structures as opposed to shorter peptides, such as a 15-mer peptide. This capacity of peptides to adopt defined secondary and/or tertiary structures coded by those phages containing multiple or triple DNA inserts may in-turn account for the selection of these types of phages from random phage display libraries during selection or panning procedures.

Different transport mechanisms operate in epithelial cells. Some transport mechanisms are carrier mediated, whereby a carrier or receptor will bind to a ligand and transport the bound ligand into or through the epithelial cell. Other transport systems operate by transcytosis, whereby a carrier or receptor site will bind a ligand, the carrier: ligand complex is internalized by endocytosis and thus delivers a ligand (or drug) into or through the cell.

Some of the peptides disclosed by this invention were discovered by use of an *in vivo* random screening approach that identified peptide sequences that interacted with undefined or unknown receptor/carrier sites in tissues, such as epithelial cells. This screening approach included contacting a predetermined amount of phage from a random phage library or a preselected phage library with a first side of a tissue barrier *in vivo* (such as injecting phage into the lumen of an animal), harvesting phage which is transported to the opposite side of the tissue barrier to select transported phage (such as harvesting phage from brain tissue), amplifying the transported phage in a host and identifying at least one random peptide coded by a transported phage to identify a peptide which permits or facilitates the transport of an active agent through a human or animal tissue. If desired, the contacting, harvesting and amplifying steps can be repeated a predetermined number of times using the transported phage obtained in the previous cycle.

Subsequently, the corresponding peptide sequences coded by the selected phages, obtained by the procedures above and identified following DNA sequencing of the appropriate gene III or gene VIII genes of the phage, were synthesized. The binding and transport of the synthetic peptide itself across the model cell culture or isolated tissue system (such as colonic) permits direct assessment of the transport

characteristics of each individual peptide. In addition, fusion of the selected peptide(s) sequences with other peptides or proteins permits direct assessment of the transport of such chimeric proteins or peptides across the model systems. Such chimeric proteins or peptides can be synthesized either *in vitro* or by conventional recombinant technology techniques whereby the cDNA coding for the transporting peptide and the cDNA coding for the drug peptide or protein are ligated together in-frame and are cloned into an expression vector which in turn will permit expression in the desired host, be it prokaryotic cells or eukaryotic cells or transgenic animals or transgenic plants. For instance, the cDNAs coding for the modified NH_2-terminal region of the pIII proteins can be subcloned into the genes or cDNAs coding for selected protein molecules (e.g., calcitonin, insulin, interferons, interleukines, cytokines, EPO, colony stimulating factors etc.) and these modified genes or cDNAs can be expressed in *E. coli* or suitable mammalian cells or transgenic animals or transgenic plants. The expressed recombinant proteins can be purified and their transcellular, carrier-mediated, transcytotic and/or paracellular transport across human or animal tissue can be verified.

In addition, the transporting peptides can be used to coat the surface of nanoparticulate or microparticulate drug delivery vehicles. Such coatings can be performed by either direct adsorption of the peptide to the surface of the particulate system or alternatively by covalent coupling of the peptide to the surface of the particulate system, either directly or via a linking moiety or by covalent coupling of the peptide to the polymers used in the production of nanoparticulate or microparticulate drug delivery vehicles, followed by the utilization of such peptide:polymer conjugates in the production of nanoparticulate or microparticulate drug delivery vehicles.

Description and Preparation of Phage Display Libraries

D38 and DC43 are random phage display libraries in which gene III codes for random peptides of 38 and 43 residues in size, respectively. These libraries are described in McConnell et al, *Molecular Diversity* 1:154-176 (1995), US Serial No.

310,192 filed September 21, 1994, US Serial No. 488,161 filed June 7, 1995, and WO 96/09411, which references are hereby incorporated by reference.

A large scale preparation of each of the bacteriophage libraries was made in the *E. coli* host strain K91Kan. A single K91Kan colony was innoculated into a sterile 50 ml tube containing 20 ml LB broth (Yeast extract (Gibco) - 1 g; Tryptone (Gibco) - 2 g; NaCl - 1 g; and distilled water - 200 ml) together with kanomycin (final concentration 100μg/ml) and grown to mid log phase with 200 rpm agitation at 37°C (OD 0.45 at 600 nm). The cells were allowed to incubate with gentle shaking (100 rpm, 37° C) for 5 min to regenerate sheared F pili. The cells were pelleted by centrifugation at 2200 rpm for 10 min at room temperature, the supernatant removed and the cells gently resuspended in 20 ml 80 mM NaCl and shaken gently (100 rpm, 37° C) for 45 min. The cells were centrifuged again and the cell pellet was gently resuspended in 1 ml cold NAP buffer (NaCl (5 M stock) - 1.6 ml; $NH_4H_2PO_4$ (0.5 M stock, pH 7.0) - 10 ml; and distilled water - 88.4 ml). The cells were stored at 4°C and remained infectable for 3-5 days.

The primary libraries were amplified by inoculating two 1 l flasks containing 100 ml terrific broth with 1 ml of an overnight culture of K91Kan cells (grown in LB + 100 μg/ml kanamycin). This culture was incubated at 37°C and 200 rpm until the OD_{600} of a 1:10 dilution was 0.2 and then further incubated for 5 min at 37°C and 200 rpm to allow sheared F pili to regenerate. 10 μl of the primary library was added to each flask with continued slow shaking for 15 min. Each culture was poured into a prewarmed 2 l flask containing 1 l LB + 0.22 μg/ml tetracycline and shaken at 200 rpm for 35 min. 1 ml of 20 mg/ml tetracycline was added and 7 μl samples were removed from each flask. The flasks were replaced in an incubator with continued shaking overnight. 200 μl of various serial dilutions (10^{-4}, 10^{-6}, 10^{-8}, 10^{-10} dilutions) of each culture were spread on LB + 40 μg/ml tetracycline and 100 μg/ml kanamycin plates and incubated overnight. The colonies were counted.

Large scale purification of phage was accomplished by dividing the culture evenly between two 500 ml centrifuge tubes and centrifuging at 5,000 rpm for 10 min at 4°C. The supernatants were transferred to fresh tubes and recentrifuged at 8,000 rpm for 10 min at 4°C. The final cleared supernatants were poured into fresh tubes

and the net volume was noted. 0.15 vol PEG/NaCl (PEG 8000 - 100 g; NaCl - 116.9 g; and distilled water - 475 ml) was added and the tubes were mixed gently by inversion (X 100 times) and stored on ice for >4 h (or overnight at 4°C). Following centrifugation at 8,000 rpm for 40 min at 4°C, the supernatant was decanted, recentrifuged briefly and residual supernatant was removed by pipetting. 10 ml TBS (Tris HCl (pH 7.5) - 0.60 g; NaCl - 0.88 g; and distilled water - 100 ml) was added and the tube was incubated at 37°C and 200 rpm for 30 min to dissolve pellet. The tube was centrifuged briefly and the solutions from both tubes were transferred to a single Oak Ridge tube, centrifuged at 10-15,000 rpm for 10 min at 4°C and the supernatant was removed to a fresh tube. 0.15 vol PEG/NaCl was added and the phage were allowed to precipitate on ice for 1 h. The procedures from the addition of 10 ml TBS were repeated. Into a tared 30 ml Beckman polyallomer tube, 4.83 g CsCl was added, the tube retared and the phage solution was added. TBS was added to a net weight of 10.75 g (total volume 12 ml of a 31% w/w solution of CsCl, density 1.3 g/ml). A ratio of 31:69 w/w ratio is essential. Following centrifugation in the ultracentrifuge at 20,000 rpm and 4°C for 48 h, the tube was illuminated from the top with a visible light source and identify the phage band:

Phage band - upper band, approximately 5 mm, faint, blue, non-flocculent

PEG - lower band, narrow, stringy, flocculent, opaque, white

The fluid was aspirated off to 2 mm above the phage band and the phage band was withdrawn using a sterile wide aperture transfer pipette and placed in a 26 ml polycarbonate centrifuge tube. The tube was filled to the shoulder with TBS, mixed and centrifuged at 50,000 rpm for 4 h at 4°C in the 60Ti rotor (repeated). The pellet was dissolved in 10 ml TBS by gentle vortexing and allowed to soften overnight in the cold and revortexed (repeated). The pellet was then dissolved in TBS (2 ml per litre of original culture) by vortexing, allowed to soften overnight at 4°C and revortexed. The tube was centrifuged briefly to drive solution to the bottom of the tube and transferred to 1.5 ml microtubes. Sodium azide (0.02%) can be added and the solution can be heated to 70°C for 20 min to kill residual microorganisms. Following microfuging for 1 min to clear the solution, the supernatant was transferred to sterile microtubes and stored at 4°C. 200 µl of a 1:100 dilution was scanned from 240-320

nm to determine the concentration of physical particles and titre 10 µl of a 10^{-8} dilution on 10 µl of starved K91Kan cells. 200 µl of the infections was spread on LB (+ 40 µg/ml tetracycline and 100 µg/ml kanamycin) plates, incubated at 37 °C for 24 h and counted the number of colonies to determine the titre of infectious units in the phage stocks.

Culturing of Caco-2

The Caco-2 (ATCC designation: CCL 248; derived from a lung metastasis of a colon carcinoma in a 72-year old male) were cultured initially in $25cm^2$ flasks, until they reached confluency. T84 cells were grown in 1:1 DMEM:Ham's F12 medium containing 2mM glutamine, 15mM HEPES, 10% fetal calf serum (FCS), 1 % MEM non essential amino acids and 50U ml^{-1} penicillin and 50µg ml^{-1} streptomycin. Caco-2 cells were grown in DMEM + glutamax-1 containing 10% FCS, 1% MEM non essential amino acids, 50U ml^{-1} penicillin and 50µg ml^{-1} streptomycin. All cells were incubated at 37°C in 95% O_2 / 5% CO_2. At confluence the cells were used to seed snapwells.

The seeding of snapwells was essentially as follows for T-84 cells (a concentration of 1 x 10^6 cells/1.0ml of medium is required for each 12mm snapwell; a 100% confluent flask of T84 contains approximately 8 x 10^6 cells and would be sufficient to seed 8 snapwells). The flasks were trypsinised and cells were carefully resuspended, making sure there are no clumps or air bubbles. 2.6ml of tissue culture medium is placed in the bottom of the wells and 0.1ml on the filter and placed in the incubator for 10 mins at 37°C. 1.5ml of the cell suspension was added to each filter, being careful not to let any fall into the bottom of the well. The filter was placed back in the incubator and checked after 24 hrs. The cells were routinely monitored for adequate TER using an EVOM chopstick epithelial voltometer (WPI). In the case of Caco-2 cells, the seeding of Caco-2 cells was essentially the same as for T-84 cells except that they are seeded at 5 x 10^5 rather than 1 x 10^6 cells/ snapwell.

The subsequent maintenance and feeding of the cells on the snapwells was as follows: when feeding the wells, the medium was removed from the basolateral side of the snapwell first. The medium was removed from the monolayer with a pipette

being careful not to touch the filter and then 1ml of growth medium was place onto the apical side and 2ml of growth medium into the basolateral side. Spillages of medium on the sides of the plate outside the well were checked for and swabbed with a cotton bud moistened with alcohol if necessary. Following seeding on the snap wells, the cells were fed on a daily basis and were cultured on the snapwells for between 21-30 days, during which time the cells spontaneously differentiated and become polarized.

Enzyme linked immuno-sorbent assay (ELISA) for fd-derived phage on Caco-2 cells

Caco-2 cells (100μl) were grown to confluence in 96 well tissue culture plates (2×10^5 cells/well grown for 2 days in growth medium containing DMEM/Glutamax + 1% Pen/Strep, 1% MEM & 10% FCS). After two days growth, 100 μl of 10% formaldehyde [Formaldehyde (38%) sterile distilled water (1: 3 vol)] was added to the confluent Caco-2 cell monolayers followed by incubation for 15 min at room temperature. The contents of the microtitre wells was emptied by inversion/flicking and the wells were washed three times with DPBS (Dulbecco's PBS). Each well was filled with 200 μl of 0.1% phenylhydrazine-DPBS (0.1% phenylhydrazine in DPBS) and incubated for 1 h at 37°C. Subsequently, the contents of the microtitre wells were emptied by inversion/flicking and the wells were washed three times with DPBS. 200 μl of 0.5% BSA in DPBS was added to each well followed by incubation for 1 h at room temperature. Each well was next washed three times in 1% BPT (1% BSA, 0.05% Tween 20 in DPBS).

Phage samples (100 μl in 1% BPT) (either neat phage at 10^{10} pfu/ml or 1:25 or 1:100 dilutions thereof) were added to the wells, followed by incubation at room temperature for 2 h. The contents of the microtitre wells were removed by inversion/flicking and the wells were washed five times in 1% BPT. 100 μl of horse-radish peroxidase (HRP) -anti-M13 conjugate (HRP/anti-M13 conjugate:horseradish peroxidase conjugated to sheep anti-M13 IgG; 1:5000 working dilution in 1% BPT; Pharmacia 27-9402-01) was added to to each well, followed by incubation for 1 h at room temperature. The contents of the microtitre wells were again removed by inversion/flicking and the wells were washed five times in 1% BPT. 200 μl of TMB

substrate solution (3,3',5',5-tetramethylbenzidine; Microwell Peroxidase Substrate System; Kirkegaard & Perry Laboratories CN 50-76-00; prepared by mixing equal amounts of TMB Peroxidase Substrate A and Peroxidase Solution B in a glass container immediately before use) was added to each well, followed by incubation at room temperature for 20-60 min. Thereafter, absorbance readings were read at 650 nm on a microtitre plate reader.

Processing of harvesting site tissue

Harvesting site tissue, such as brain, heart, kidney, spleen, liver, pancreas. duodenum or ileum tissue, is collected from animals following introduction *in vivo* of the phage display library at a site separated from the harvesting site by a tissue barrier. The animals are sacrificed at a predetermined time following administration of the library, the tissue is removed and the tissue is either processed immediately or frozen in liquid nitrogen (stored at -80°C) for processing at a later date. The tissue samples are homogenised in PBS containing protease inhibitors and the homogenate is used to infect *E.coli,* thus permitting amplification of phages that were transported to the harvesting site tissue.

Processing of tissue adjacent the phage display library administration site

For use in the *in vivo* screening approach described herein, the phage display library is purified such as by either PEG precipitation or by sucrose or CsCl density centrifugation. The phage display library is resuspended in PBS (or TBS) buffer and injected into the *in vivo* animal site, such as duodenum, jejunum, ileum, colon, ascending colon, transverse colon, descending colon, pelvic colon in the closed (or open) animal (rat, rabbit or other species) loop model. Following administration of the phage display library to the gastro-intestinal tract of the animal model, and withdrawal of portal and/or systemic blood samples at predetermined time points (such as 0 min, 15 min, 30 min, 45 min, 60 min up to 6 hours), or incubation of the administered phage display library in the closed (or open) loop model for a predetermined period of time, the corresponding region of the GIT track exposed to or incubated with the phage display library can be recovered at the end of the

experiments. Following repeated washings of the recovered intestinal tissue in suitable buffers such as PBS containing protease inhibitors, the washed tissue is homogenised in PBS containing protease inhibitors and the homogenate is used to infect *E.coli,* thus permitting amplification of phages which can bind tightly to the intestinal tissue. Alternatively, the recovered intestinal tissue can be homogenised in suitable PBS buffers, washed repeatedly and the phage present in the final tissue homogenate can be amplified in *E.coli.* This latter approach also permits amplification of phages which either bind tightly to the intestinal tissue or which are internalized by the epithelial cells of the intestinal tissue

Treatment of animal tissue barriers in vivo with phage display populations

The purified phage display library (random or preselected) is diluted to 500 μl in PBS buffer and injected into the closed (or open) intestinal loop model (e.g., rat, rabbit or other species). At time 0 and at successive time points after injection, a sample of either the portal circulation or systemic circulation is withdrawn. An aliquot of the withdrawn blood can be incubated with *E. coli,* followed by plating for phage plaques or for transduction units or for colonies where the phage codes for resistance to antibiotics such as tetracycline. The remainder of the withdrawn blood sample (up to 150 μl) is incubated with 250 μl of *E. coli* and 5 ml of LB medium or other suitable growth medium. The *E. coli* cultures are incubated overnight by incubation at 37°C on a shaking platform. Blood samples taken at other time points (such as 15 min, 30 min, 45 min, 60 min up to 6 hours) are processed in a similar manner, permitting amplification of phages present in the portal or systemic circulation in *E. coli.* at these times. Following amplification, the amplified phage is recovered by PEG precipitation and resuspended in PBS buffer or TBS buffer. In addition, the titer of the amplified phage, before and after PEG precipitation is determined. The amplified, PEG precipitated phage is diluted to a known phage titer (generally between 10^8 and 10^{10} phage or plaque forming units per ml) and is injected into the GIT of the animal closed (or open) loop model. Blood samples are collected from portal and/or systemic ciruclation at various time points and the phage transported into the blood samples are amplified in *E. coli* as given above for the first

cycle. Subsequently, the phage are PEG precipitated, resuspended, titered, diluted and injected into the GIT of the animal closed (or open) loop model. This procedure of phage injection followed by collection of portal and/or systemic blood samples and amplification of phage transported into these blood samples can be repeated, for example, up to 10 times, to permit the selection of phages which are preferentially transported from the GIT into the portal and/or systemic circulation.

Additionally or alternatively, at the conclusion of the portal blood sampling (typically within 60 min from administration of the phage display library) or systemic blood sampling (typically within 360 min from administration of the phage display library) or at other convenient predetermined times, the animal can be sacrificed and the harvesting site tissue, such as brain tissue, removed. The tissue can be processed immediately or frozen in liquid nitrogen (stored at -80°C) for processing at a later date. Following homogenization of the tissue in PBS containing protease inhibitors, serial dilutions (such as neat, 10^{-2}, 10^{-4}, 10^{-6} dilutions) of the tissue homogenate are titered in *E. coli*. An aliquot (100 μl) of the tissue homgentate is added to 100 μl of *E. coli* K91 Kan starved bacteria, and incubated at 37°C for 10 min followed by addition of 5 ml of LB medium or other suitable growth medium. The *E. coli* cultures are incubated overnight at 37°C and serial dilutions of amplified phage are then titered in *E. coli*. As above, the recovered phage can be administered to other animals, harvesting site tissue can be collected, and the phage transported into the tissues can be amplified repeatedly, for example, up to 10 times, to permit the selection of phages which are preferentially transported from the GIT into the harvesting site tissue.

Treatment of tissue culture cell monolayers with phage display populations

In a laminar flow cabinet, 100 μl of phage solution was mixed with 900 μl of growth medium without antibiotic (the complete recommended medium for each cell line but with no antibiotics added) in a microfuge tube. The experiment was carried out in duplicate and included a control treatment containing no phage. The TER was measured for each snapwell, noting the age of the cells and the passage number. Only intact monolayers of recommended age were used which had expected TER. The basolateral medium was replaced in the snapwells with medium without antibiotic and

the apical medium was removed. The phage solutions and control solutions were added to the apical side of the cells and the snapwell cultures were incubated as normal. At each harvest time point (e.g., 1 h, 5 h, 24 h after application of phage), the medium was removed from the basolateral side and stored in a sterile 2 ml screwcap tube at 4°C. At each time that the basolateral medium is removed, the medium was replaced with fresh medium without antibiotic. When the experiments are finished, the TER was measured and the monolayers were treated with Vircon disinfectant as per normal.

Phage/Particle Binding to Caco-2 Cells

Phage expressing presumed GIT binding peptide inserts were assayed by ELISA on fixed Caco-2 cells as follows. Cells were plated at 1 x 10^5 cells/well on 100 μl culture media and incubated at 30°C in 5% CO_2 overnight. 100 μl 25% formaldehyde was added to each well for 15 minutes. Contents of the wells were removed by inverting the plate. The plate was then washed 3 times with DPBS. 0.1% phenylhydrazine DPBS solution was added to each well and incubated for 1 hr at 37°C. The plate was inverted and washed 3 times. The plate was blocked with 0.5% BBA-DPBS for 1 hr at room temperature. The plate was inverted and washed 3 times with 1% BPT. Phage diluted with 1% BPT was added to wells containing fixed cells. Wells without phage added were used to determine background binding of the HRP conjugate. The plates were incubated 2-3 hours on a rotor at room temperature. Plates were washed as before. Plates were incubated with dilute anti M13-HRP antibody in 1% BPF for 1 hour at room temperature. Following washing, TMB substrate was added and absorbance of the plates were read at 650 nm..

Nanoparticles were added to the cells at 10mg/ml in 100μl 1%BSA-PBS (no Tween80 is used in this assay) and 2-fold serially-diluted. The 96-well plates were incubated for 1h at room temperature. The plates were washed 5 times with 1% BSA-PBS and 100μl of anti-dansyl antibody (Cytogen DB3-226.3; 0.5 μg/ml; batch May 1997) was added per well and the plates incubated 1h at room temperature. The wells were washed 5 times with 1% BSA-PBS and 100μl of goat anti-mouse γ:HRP antibody (Southern Biotechnology CN. 1060-05; 1:10,000) was added per well and

the plates incubated 1h at room temperature. After washing 5 times with 1% BSA-PBS, 100μl of TMB peroxidase substrate (KPL CN. 50-76-00) was added to the wells and the optical density at 650nm was measured after 15 minutes.

Binding of Coated Particles to fixed Caco-2 Cells

Binding of nanoparticles coated with targeting peptides to fixed Caco-2 cells was investigated using an ELISA assay based on reaction of antibody with the dansyl moiety present on the peptides. Confluent Caco-2 monolayers grown in 96-well plates (p38) were fixed and treated with 0.1% phenylhydrazine before blocking with 0.1% BSA in PBS. Control and dansyl peptide-coated nanoparticles were resuspended in sterile water at 10mg/ml and stirred with a magnet for 1h at room temperature.

Formulations: General Method for Preparation of Coacervated Particles.

Solid particles containing a Therapeutic as defined herein are prepared using a coacervation method. The particles have a particle size of between about 10nm and 500 μm, most preferably 50 to 800 nm. In addition the particles contain targeting ligands which are incorporated into the particles using a number of methods.

Typically, particles are formed using the following general method:

An aqueous solution (A) of a polymer, surface active agent, surface stabilising or modifying agent or salt, or surfactant preferably a polyvinyl alcohol (PVA) or derivative with a % hydrolysis 50 - 100% and a molecular weight range 500 - 500,000, most preferably 80-100% hydrolysis and 10,000-150,000 molecular weight, is introduced into a vessel. The mixture (A) is stirred under low shear conditions at 10- 2000 rpm, preferably 100-600 rpm. The pH and/or ionic strength of this solution may be modified using salts, buffers or other modifying agents. The viscosity of this solution may be modified using polymers, salts, or other viscosity enhancing or modifying agents.

A polymer, preferably poly(lacide-co-glycolide) (hereinafter PLGA), polylactide, polyglycolide or a combination thereof or in any enantiomeric form or a covalent conjugate of the these polymers with a targeting ligand is dissolved in water

miscible organic solvents to form organic phase (B). Most preferably a combination of acetone and ethanol is used in a range of ratios from 0:100 acetone: ethanol to 100: 0 acetone: ethanol depending upon the polymer used.

Additional polymer(s), peptide(s) sugars, salts, natural/biological polymers or other agents may also be added to the organic phase (B) to modify the physical and chemical properties of the resultant particle product.

A drug or bioactive substance may be introduced into either the aqueous phase (A) or the organic phase (B). A targeting ligand may also be introduced into either the aqueous phase (A) or the organic phase (B) at this point.

The organic phase (B) is added into the stirred aqueous phase (A) at a continuous rate. The solvent is evaporated, preferably by a rise in temperature over ambient and/or the use of a vacuum pump. The particles are now present as a suspension (C). A targeting ligand may be introduced into the stirred suspension at this point.

A secondary layer of polymer(s), peptide(s) sugars, salts, natural/biological polymers or other agents may be deposited on to the pre-formed particulate core by any suitable method at this stage.

The particles (D) are then separated from the suspension (C) using standard colloidal separation techniques, preferably by centrifugation at high 'g' force, filtration, gel permeation chromatography, affinity chromatography or charge separation techniques. The supernatant is discarded and the particles (D) re-suspended in a washing solution (E) preferably water, salt solution, buffer or organic solvent(s). The particles (D) are separated from the washing liquid in a similar manner to previously described and re-washed, commonly twice. A targeting ligand may be dissolved in washing solution (E) at the final washing stage and may be used to wash the particles (D).

The particles may then be dried. Particles may then be further processed for example, tabletted, encapsulated or spray dried.

The release profile of the particles formed above may be varied from immediate to controlled or delayed release dependent upon the formulation used and/or desired.

Drug loading may be in the range 0-90% w/w. Targeting ligand loading may be in the range 0-90% w/w.

The surface active agent of aqueous solution (A) in the general method above includes agents that may be referred to as emulsifing agents, detergents, solubilizing agents, wetting agents, foaming and antifoaming agents, flocculants and defloculant. Representative examples include anionic surface active agents such as sodium dodecanoate, sodium dodecyl(lauryl)sulphate, sodium dioctyl sulphosuccinate, cetostearyl alcohol, stearic acid and its salts such as magnesium stearate and sodium stearate, sodium dodecyl benzene sulphonate, sodium cholate triethanolamine; cationic surface active agents such as hexadecyl trimethyl ammonium bromide(cetrimide), dodecyl pyridinium iodide, dodecyl pyridinium chloride; non-ionic suface active agents such as hexaoxyethylene monohexadecyl ether, polysorbates(Tweens), sorbitan esters(Spans), Macrogol ethers, Poloxalkols (Poloxamers),PVA, PVP, glycols and glycerol esters, fatty alcohol poly glycol ethers, dextrans, higher fatty alcohols; and amphoteric surface active agent such as N-dodecyl alanine, lecithin, proteins, peptides, polysaccharides, semisynthetic polysaccharides, sterol-containing substances, and finely divided solids such as magnesium hydroxide and montmorillonite clays.

The organic phase (B) polymer of the general method given above may be soluble, permeable, impermeable, biodegradable or gastroretentive. The polymer may consist of a mixture of polymer or copolymers and may be a natural or synthetic polymer. Representative biodegradable polymers include without limitation polyglycolides; polylactides; poly(lactide-co-glycolides), including DL, L and D forms; copolyoxalates; polycaprolactone; polyesteramides; polyorthoesters; polyanhydrides; polyalkylcyanoacrylates; polyhydroxybutyrates; polyurethanes; albumin; casein; citosan derivatives; gelatin; acacia; celluloses; polysaccharides; alginic acid; polypeptides; and the like, copolymers thereof, mixtures thereof and stereoisomers thereof. Representative synthetic polymers include alkyl celluloses; hydroxalkyl celluloses; cellulose ethers; cellulose esters; nitro celluloses; polymers of acrylic and methacrylic acids and esters thereof; dextrans; polyamides; polycarbonates; polyalkylenes; polyalkylene glycols; polyalkylene oxides;

polyalkylene terephthalates; polyvinyl alcohols; polyvinyl ethers; polyvinyl esters; polyvinyl halides; poyvinylpyrrolidone; polysiloxanes and polyurethanes and co-polymers thereof.

Specific examples include the following examples:

<u>Formulation 1: Peptide added at the final washing stage</u>

Product: Bovine Insulin loaded nanoparticles

Aim: To prepare a 2g batch of insulin loaded nanoparticles at a theoretical loading of 50mg/g and with the peptide 41.1 added.

Formulation Details

RG504H (PLGA; Boehringer Ingleheim)	2.0g
Acetone	45mls
Ethanol:	5mls
PVA (aq. 5%w/w)	400mls
Bovine Insulin (Lot no. 86H0674)	100mg
Peptide: peptide 41.1	10mg/50ml dH_2O

Experimental details:

The 5% w/v PVA solution is prepared by heating water until near boiling point, adding PVA and stirring until cool. The organic phase is prepared by adding acetone, 45mls, and ethanol, 5mls, together. The polymer solution is prepared by adding RG504H, 2g, to the organic phase and stirring until dissolved. The IKA reactor vessel is set up, all seals greased and the temperature is set at 25°C. The PVA solution, 400mls, is added into the reactor vessel and stirred at 400 rpm.

Bovine insulin 100mg, is added into the stirring PVA solution. Using clean tubing and a green needle the polymer solution, is slowly dripped in the stirring PVA solution with the peristaltic pump set at 40. The solvent is allowed to evaporate by opening the ports and allowing the dispersion to stir overnight at 400 rpm.

The suspension is centrifuged in a Beckman Ultracentrifuge with swing-out rotor at 12,500 rpm and 4°C. The supernatant is decanted and discarded and the

"cake" of particles is broken up. dH_2O (200mls) is added to wash the particles. The centrifugation and washing steps were repeated twice.

The peptide solution, (peptide 41.1 10mg) in 50ml dH_2O is prepared and added to the particles for a final washing stage. The suspended particles were centrifuged as before. The supernatant liquid is decanted off, the 'cake' broken up and the particles were dried in the vacuum oven.

The particles were ground, placed in a securitainer and sent for analysis.

Formulation 2: Peptide added at the beginning of manufacture

Product: Bovine Insulin loaded nanoparticles

Aim: To prepare a 2g batch of insulin loaded nanoparticles at a theoretical loading of 50mg/g and with the dynamin 41.2 added at the beginning of manufacture.

Formulation Details

RG504H	(Lot no. 250583)	2.0g
Acetone		5mls
Ethanol:		5mls
PVA(aq. 5%w/w)		400mls
Bovine Insulin (Lot no. 65H0640)		100mg
Peptide: dynamin 41.2		10mg

Experimental details:

The 5% w/v PVA solution is prepared by heating water until near boiling point, adding PVA and stirring until cool. The organic phase is prepared by adding acetone, 45mls, and ethanol, 5mls, together. The polymer solution is prepared by adding RG504H, 2g, to the organic phase prepared in step above and stirring until dissolved. The IKA reactor vessel is set up, all seals greased and the temperature is set at 25°C. The PVA solution, 400mls, is added into the reactor vessel and stirred at 400 rpm.

Bovine insulin 100mg, is added into the stirring PVA solution. PAX 2 (ZELAN018ii 10mg) is added to the stirring PVA solution. Using clean tubing and a green needle the polymer solution, (step 3), is slowly dripped in the stirring PVA

solution with the peristaltic pump set at 40. The solvent is allowed to evaporate by opening the ports and allowing the dispersion to stir overnight at 400 rpm. The suspension is centrifuged in a Beckman Ultracentrifuge with swing-out rotor at 12,500 rpm and 4°C. The supernatant is decanted and discarded.

The "cake" of particles is broken up and dH_2O (200mls) is added to wash the particles. The centrifugation and washing steps were repeated twice. The 'cake' is broken up and the particles were dried in the vacuum oven.

The particles were ground, placed in a securitainer and sent for analysis.

Formulation 3: Peptide added 1 hour before centrifugation

Product: Bovine Insulin loaded nanoparticles

Aim: To prepare a 1g batch of insulin loaded nanoparticles at a theoretical loading of 50mg/g and with the peptide 41.1 added 1 hour before centrifugation.

Formulation Details

RG504H	(Lot no. 250583)	1.0g
Acetone		22.5mls
Ethanol:		2.5mls
PVA(aq. 5%w/w)		200mls
Bovine Insulin (Lot no. 65H0640)		50mg
Peptide: peptide 41.1		5mg

Experimental details:

The 5% w/v PVA solution is prepared by heating water until near boiling point, adding PVA and stirring until cool. The organic phase is prepared by adding acetone, 22.5mls, and ethanol, 2.5mls, together. The polymer solution is prepared by adding RG504H, 1g, to the organic phase prepared above and stirring until dissolved. The IKA reactor vessel is set up, all seals greased and the temperature is set at 25°C. The PVA solution, 200mls, is added into the reactor vessel and stirred at 400 rpm.

Bovine insulin 50mg, is added into the stirring PVA solution. Using clean tubing and a green needle the polymer solution, is slowly dripped in the stirring PVA

solution with the peristaltic pump set at 40. The solvent is allowed to evaporate by opening the ports and allowing the dispersion to stir overnight at 400 rpm.

Peptide 41.1 is added to the stirring particle suspension. After 1 hr the suspension is centrifuged in a Beckman Ultracentrifuge with swing-out rotor at 12,500 rpm and 4°C. The supernatant is decanted and discarded. The "cake" of particles is broken up and dH_2O (200mls) is added to wash the particles. The centrifugation and washing steps are repeated twice.

The 'cake' is broken up and the particles were dried in the vacuum oven. The particles were ground, placed in a securitainer and sent for analysis.

Formulation 4: Leuprolide acetate loaded nanoparticles

Aim: To prepare a 3g batch of leuprolide-acetate loaded nanoparticles at a theoretical loading of 20mg/g and with the peptide 41.1 added.

Formulation Details

RG504H	(Lot no. 271077)	3.0g
Acetone		67.5mls
Ethanol:		7.5mls
PVA(aq. 5%w/w)		600mls
Leuprolide acetate	(Lot no. V14094)	60mg
Peptide: P31 (Zelan024)		15mg/50ml dH_2O

Experimental details:

The PVA solution is prepared and the organic phase is prepared by adding acetone, 67.5mls, and ethanol, 7.5mls, together. The polymer solution is prepared by adding RG504H, 3g, to the organic phase prepared above and stirring until dissolved. The IKA reactor vessel is set up, all seals greased and the temperature is set at 25°C. The PVA solution, 600mls, is added into the reactor vessel and stirred at 400 rpm.

Leuprolide acetate, 60mg, is added into the stirring PVA solution. Using clean tubing and a green needle the polymer solution, is slowly dripped in the stirring PVA solution with the peristaltic pump set at 40. The solvent is allowed to evaporate by opening the ports and allowing the dispersion to stir overnight at 400 rpm. The

suspension is centrifuged in a Beckman Ultracentrifuge with swing-out rotor at 15 rpm and 4°C. The supernatant is decanted and retained for analysis.

The "cake" of particles is broken up and dH_2O 200mls) is added to wash the particles. The centrifugation and washing steps were repeated twice.

The peptide solution, (peptide 41.1, 15mg) in 50ml dH_2O is prepared and added to the particles for a final washing stage. The suspended particles were centrifuged as before. The supernatant liquid is decanted off and the particles were dried in the vacuum oven.

The particles were ground, placed in a securitainer and sent for analysis.

<u>Formulation 5: Peptide added by 'spiking' polymer phase with polymer-peptide conjugate</u>

Product: Bovine Insulin loaded nanoparticles

Aim: To prepare a 3g batch of insulin loaded nanoparticles at a theoretical loading of 50mg/g and with the polymer-peptide conjugate PLGA-peptide 41.1 added.

Formulation Details

RG504H Lot no. 271077)	2.85g
RG504H-peptide 41.1 conjugate	0.15g
Acetone	67.5mls
Ethanol:	7.5mls
PVA(aq. 5%w/w)	600mls
Bovine Insulin(Lot no. 86H0674)	150mg

Experimental details:

The 5% w/v PVA solution is prepared by heating water until near boiling point, adding PVA and stirring until cool. The organic phase is prepared by adding acetone, 67.5mls, and ethanol, 7.5mls, together. The polymer solution is prepared by adding RG504H, and the polymer -peptide conjugate, to the organic phase prepared in step 2 and stirring until dissolved.

The IKA reactor vessel is set up, all seals greased and the temperature is set at 25°C. The PVA solution, 400mls, is added into the reactor vessel and stirred at 400 rpm.

Bovine insulin 100mg, is added into the stirring PVA solution. Using clean tubing and a green needle the polymer solution, (step 3), is slowly dripped in the stirring PVA solution with the peristaltic pump set at 40. The solvent is allowed to evaporate by opening the ports and allowing the dispersion to stir overnight at 400 rpm.

The suspension is centrifuged in a Beckman Ultracentrifuge with swing-out rotor at 12,500 rpm and 4°C. The supernatant is decanted and discarded. The "cake" of particles is broken up and dH_2O (200mls) is added to wash the particles. The centrifugation washing step is repeated twice.

The 'cake' is broken up and the particles were dried in the vacuum oven. The particles were ground, placed in a securitainer and sent for analysis.

Example 1: Transport of Phage from Rat Lumen into the Portal and Systemic Circulation

In this study, phage from random phage display libraries as well as control phage were injected into the lumen of the rat gastro-intestinal tract (*in situ* rat closed loop model). Blood was collected over time from either the systemic circulation or portal circulation and the number of phage which were transported to the circulation was determined by titering blood samples in *E. coli*. At the conclusion of the collection of either systemic (300 min) or portal blood (60 min), the animals were sacrificed and brain, heart, kidney, spleen, ileum, duodenum, liver and pancreas tissue samples were removed, frozen in liquid nitrogen and stored at -80°C.

The phage display libraries used in this study were D38 and DC43 in which gene III codes for random 38-mer and 43-mer peptides, respectively. As a negative control, the identical phage M13mp18, in which gene III does not code for a "random" peptide sequence, was used. Both the library phages D38 and DC43 were prepared from *E.coli*, mixed together, dialyzed against PBS, precipitated using PEG/NaCl and were resuspended in PBS buffer. The M13mp18 control was

processed in a similar manner. The titer of each phage sample was determined and the phage samples were diluted in PBS to approximately the same titers prior to injection into the rat closed loop model.

For sampling from the systemic circulation, approximately 15 cm of the duodenum of Wistar rats was tied off (closed loop model), approximately 0.5ml of phage solution was injected into the closed loop and blood (0.4ml) was sampled from the tail vein at various times. The time points used (in min) were: 0, 15, 30, 45, 60, 90, 120, 180, 240 and 300 minutes. For sampling from the portal circulation, the portal vein was catheterized, approximately 15 cm of the duodenum was tied off (closed loop model), 0.5ml of phage solution was injected into the closed loop and blood was sampled from the portal vein catheter at various times. As the portal sampling is delicate, sampling times were restricted to 15, 30, 45 and 60 minutes, where possible. The volume of phage injected into each animal is shown in Table 1.

Table 1

ANIMALS (15)	VOLUME OF PHAGE INJECTED
R1-R3	0.50 ml
R4	0.43 ml
R5-R15	0.45 ml

The estimated number of transported phage has been adjusted to account for differences in volume injected into each animal (using 0.5 ml as the standard volume).

To investigate transport into the systemic circulation, animals R1, R2 and R3 received the control phage M13mp18 and animals R4, R5, R6 and R7 received the test phage D38 / DC43 mix. To investigate transport into the portal circulation, animals R8, R9 and R10 received the control phage M13mp18 and animals R11, R12, R13 and R14 received the test phage D38 / DC43 mix. Animal R15* received the combined phage samples from animals R4-R7 (see Table 8) which were sampled from the systemic circulation on day one, followed by amplifiction in *E. coli,* PEG precipitation and resuspension in PBS. On subsequent analysis, the titer of this phage

was found to 100 times greater than the other phage samples used for animals R8 - R14. Thus, the date presented for animal R15 in Table 9 is adjusted down.

Approximately 0.4 ml of the blood was collected at each time point in each model system. 30 μl of the collected blood (systemic) was mixed with 100 μl of the prepared *E.coli* strain K91Kan, incubated at 37°C for 30 min, and plated out for plaque formation using Top Agarose on LB plates. Various negative controls were included in the titering experiments. The following day the number of plaques forming units (pfu's) was determined. Similarly, 30 μl of the collected blood (portal) and serial dilutions (1:100, 1:1000) thereof was mixed with 100 μl of the prepared *E.coli* strain K91Kan, incubated at 37°C for 30 min, and plated out for plaque formation using Top Agarose on LB plates. The following day the number of plaques forming units (pfu's) was determined.

In addition, approximately 300 μl of the collected blood from each time point (systemic and portal) was incubated with 5ml of prepared *E.coli* strain K91Kan in modified growth media containing 5mM $MgCl_2$/MgSO4, incubated at 37°C overnight with shaking (to permit phage amplification). The samples were centrifuged and the cell pellet was discarded. Samples of the phage supernatant were collected, serially diluted (10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}) in TBS buffer and were plated for plaques in order to determine the number of pfu's present in the amplified phage samples.

Furthermore, an aliquot of phage was removed from the "amplified" supernatants obtained from test animals #R4-R7 (samples from each time point were used), combined and was PEG-precipitated for two hours. The precipitated phage was resuspended in PBS buffer and was injected into closed loop model of animal #R15, followed by portal sampling.

The number of phage transported from the closed loop model into the systemic circulation is presented in Table 2. The number of phage transported from the closed loop model into the portal circulation is presented in Table 3. These

numbers are corrected for phage input difference and for volume input differences. Clearly, more phage are present in the portal samples than in the systemic samples, indicative of either hepatic or RES clearance and/or phage instability in the systemic circulation. In addition, the uptake of phage from the GIT into the portal circulation is quite rapid, with substantial number of phages detected within 15 minutes. The results from the portal sampling experiments would also indicate that the kinetics of uptake of phage from the D38 / DC43 libraries is quicker than that of the control phage. Thus, there may be preferential uptake of phage coding for random peptide sequences from the GIT into the portal circulation. In the case of animals R13, R14 and R15*, the % of the phage transported into the titered blood sample within the limited time frame (30, 45 and 15 mins, respectively) is estimated as 0.13%, 1.1% and 0.013%, respectively.

Table 2: Number of phage transported from the closed loop model into the Systemic circulation

Time (min)	R1	R2	R3	R4	R5	R6	R7
0	0	0	0	0	0	0	0
15	0	1	9	0	0	1	7
30	2	1	0	0	46	1	11
45	10	4	2	1	32	0	20
60	63	19	21	1	114	0	21
90	104	20	18	3	115	0	22
120	94	24	27	0	64	0	6
180	94	12	23	1	413	0	0
240	14	1	20	0	36	0	0
300	1	1	4	2	0	0	0
Total number of transported phage	382	83	124	8	820	2	87

Animals R1, R2 and R3 received the control phage M13mp18

Animals R4, R5, R6 and R7 received the test phage D38 / DC43 mix

Table 3: Number of phage transported from the closed loop model into the Portal circulation								
Time (min)	R8	R9	R10	R11	R12	R13	R14	R15*
15	15	6	3	1	19	231,000	1,000,000	20,000
30	1	5	26	-	0	60,000	272,000	-
45	-	1	555	-	1	-	1,240,000	-
60	-	-	-	-	420,000	-	-	-

Animals R8, R9 and R10 received the control phage M13mp18

Animals R11, R12, R13 and R14 received the test phage D38 / DC43 mix

Animal R15* received the combined phage samples from animals R4-R7 (see Table 8) which were sampled from the systemic circulation on day one, followed by PEG precipitation and resuspension in PBS. On subsequent analysis, the titer of this phage was found to be 100 times greater than the other phage samples used for animals R8 - R14. Thus, the date presented for animal R15* in Table 9 is adjusted down.

These studies demonstrate that both the control phage and the D38/DC43 phages are transported over time from the lumen of the GIT into the portal and systemic circulation, as demonstrated by titering the phage transported to the blood in *E.coli*. More phage are transported from the test phage samples into the portal circulation than the corresponding control phage sample. In addition, the kinetics of transport of the test phage into the portal circulation does appear to exceed that of the control phage. Phage from the D38/DC43 libraries which appeared in the systemic circulation of different animals (R4-R7) were pooled, amplified in *E.coli*, precipitated, and re-applied to the lumen of the GIT, followed by collection in the portal circulation and titering in *E.coli*. These selected phage were also transported from the lumen of the GIT into the portal circulation. This *in situ* loop model may represent an attractive screening model in which to identify peptide sequences which facilitate transport of phage and particles from the GIT into the circulation.

The frozen brain tissues for all the animals were thawed, cut into small pieces, resuspended in 5 ml of sterile PBS containing a cocktail of protease inhibitors (Boehringer) and homogenized in an Ultrathorex homogenizer. Serial dilutions (neat and 10^{-2}, 10^{-4}, 10^{-6} dilutions) were titered in *E. coli*. K91 Kan starved bacteria. In

addition, aliquots (100 μl) of the tissue homogenate were added to 100 μl of *E. coli* K91 Kan starved bacteria, incubated at 37°C for 10 min, followed by addition of 5 ml of LB medium and incubation overnight at 37°C in a rotating incubator. Serial dilutions of amplified phage were then titered in *E. coli*. No phage was detected in tissue homogenate samples obtained from animals R4-R7 (sacrificed after systemic sampling) prior to amplification but phage were detected after amplification. Phage were detected prior to amplification in tissue honogenate samples obtained from animals R11-R14.

An equal amount of phage from animals R11 through R14 were pooled at a concentration of $5x10^{11}$ pfu/ml (in PBS). These phage were amplified as above, plated and the recombinant phage were picked and purified. Sequencing templates were prepared using the QIAprep Spin M13 columns and were sequenced using the primer SEQ. NO. ID: 17 and Sequenase Version 2.0 DNA sequencing kit. Eight DNA sequences (SEQ. NOS. ID: 1, 3, 5, 7, 9, 11, 13, 15) with corresponding peptide sequences SEQ. NOS. ID: 2, 4, 6, 8, 10, 12, 14, 16 were discovered. These peptide sequences, which were isolated because of their ability to transport phage (particles) from the GI tract to the brain, are capable of facilitating the transport of an active agent, such as a micro- or nanoencapsulated active agent, through a human or animal tissue.

<u>Example 2: Characterization of Phage Harvested from Brain Tissue</u>

Binding of the brain homing phage identified in Example 1 to fixed Caco-2 cells relative to the negative control phage M13mp18 was investigated using the procedures outlined above. The results of this study are summarized in Fig 1, which shows the binding profiles of brain selected phage to Caco-2 fixed cells. Fig. 1A Caco-2 P34, read at 30 min; Fig. A(b): Caco-2 P36, read at 20 min; and Fig. A(c); Caco-2 P34, read at 10 min. The neat sample corresponds to 10^{10} phage/well;

Fig. 2 shows the uptake and transport of brain selected phage 41.1 and 1.4 across Caco-2 cell monolayers. Fig. 2A shows the titer of phage eluted by acid

treatment of cell monolayers after 2hrs incubtion at 37°C; Fig. 2B shows the titer of cell-associated phage detected in the cell lysate after 2hrs incubation at 37°C; and Fig. 2C shows the titer of phage from the basolateral medium after 6 hrs incubation at 37°C of Caco-2 cells with phage suspension on the apical side.

Fig. 2, which illustrates the results of this experiment, shows the presence of phage in the fraction eluted with acid from the apical side of Caco-2 cell monolayers, in the cell lysate fraction, and from the basolateral side of the Caco-2 monolayers after 2hrs incubation at 37°C. The results strongly suggest that phage 41.1 and, though less strongly, phage 1.4 bind, are internalized and transported to the basolateral medium in the Caco-2 cell monolayer model.

Example 3: Characterization of Peptides Identified from Brain Homing Phage

The amino acid sequences and isoelectric points (pI) associated with various peptides identified from phage transported from the rat duodenal lumen to the brain are given in Table 4. The first amino acid of each sequence can only be either arginine (R) or serine (S) because the first two nucleotides of the coding codon (AGX) belong to the restriction site XhoI (C TCG AG) used for the cloning of random peptides in the phage DNA. If the X base is G or A the coded amino acid is arginine; if the X base is C or T the coded amino acid is serine. The sequences in bold, i.e. GCG (DC43 library) and A (D38 library), are conserved sequences used for the construction of the libraries (McConnell et al.; *Molecular Diversity* 1:165-176 (1995)).

Table 4

clone	amino acid sequence	source library	pI value	SEQ ID NO
1.4	RGYGRLAESCCVNDRCIRTVGGCGNSPASDILSNT	DC43	7.83	2
41.1	RQSAGVLGFAPTNIDDTSFHAGCGDTLAIPCRHRSSLISPARPP	DC43	8.23	16
13.1	STPGRGSGRDTGANNAADTPYANPSHRDTILSLDPSLL	D38	5.15	4
17.3	RQHLVVRDLHEPRFRDTNTGVHATFSPPVSVATDHRTPP	D38	10.37	6
18.1	SFSNLTAGDEEDDHFSGGRFNHANLTSRSHNRGQLASSA	D38	5.75	8
28.1	RQSVLDSWGGKTSVTGSLERYYASHSHTSAPTPHYASHS	D38	9.23	10
34.2	RQWVGDRADGEGNWVDEKYSRDANVISYRKHNHASQGTL	D38	7.61	12
37.1	RASDCDVECNLRWVEDVGGVWYAKTVSRMLSTT	D38	4.55	14

Table 5 itemizes the charge of the peptides at different pH as analyzed by PCGENE software.

Table 5

peptide	pH				
	4	5	6	7	8
1.4	2.26	1.23	1.00	0.82	-0.34
41.1	3.91	2.94	2.00	1.08	0.30
13.1	1.24	0.08	-0.49	-0.92	-1.06
17.3	6.53	4.91	3.02	1.35	0.94
18.1	3.19	1.15	-0.46	-1.73	-2.03
28.1	5.92	4.83	3.02	1.35	0.92
34.2	4.51	2.29	1.05	0.17	-0.10
37.1	1.21	-0.57	-0.97	-1.10	-1.72

Potential regions of homology among the peptides were analyzed leading to motifs identified in five of the eight peptides identified from the brain homing phage. Table 6 shows the relevant peptide sequence alignment; panel 6(a) highlights identical amino acids while panel 6(b) highlights conserved amino acids.

Table 6 (a)

37.1	R	A	S	D	C	D	V	E	C	N	L	R	W	V	E	D	V	G	G	V	W	Y	A
41.1	R	Q	S	A	G	V	L	G	F	A	P	T	N	I	D	D	T	S	F	H	A	G	C
28.1	R	Q	S	V	L	D	S	W	G	G	K	T	S	V	T	G	S	L	E	R	Y	Y	A
34.1	R	Q	W	V	G	D	R	A	D	G	E	G	N	W	V	D	E	K	V	S	R	D	A
17.1	R	Q	H	L	V	V	R	D	L	H	E	P	R	F	R	D	T	N	T	G	V	H	A

Table 6 (b)

37.1	R	A	S	D	C	D	V	E	C	N	L	R	W	V	E	D	V	G	G	V	W	Y	A
41.1	R	Q	S	A	G	V	L	G	F	A	P	T	N	I	D	D	T	S	F	H	A	G	C
28.1	R	Q	S	V	L	D	S	W	G	G	K	T	S	V	T	G	S	L	E	R	Y	Y	A
34.1	R	Q	W	V	G	D	R	A	D	G	E	G	N	W	V	D	E	K	V	S	R	D	A
17.1	R	Q	H	L	V	V	R	D	L	H	E	P	R	F	R	D	T	N	T	G	V	H	A

Figs. 3 and 4 show homologies of the brain-homing transport-binding peptides to known proteins. The first ten homologies obtained from a Blast search against the non-redundant SwissProt database for each peptide are given in Fig. 3. Particularly interesting homologies for peptides 41.1, 37.1 and 1.4 are reported in Fig. 4. Of particular interest is the homology of the peptide 41.1 with human, rat and murine dynamin II protein shown in Fig. 4.

Example 4: Synthetic Peptides

The peptide 41.1 and a fragment of human dynamin II homologous to it (herein termed peptide 41.2) have been synthesized and a dansyl group has been added at the N-terminal of the sequence in order to enable the detection of the peptide with anti-dansyl antibody. Additionally, fragments of peptide 41.2 were synthesized as shown in Table 7.

Table 7		
Peptide	**Description**	**Sequence**
ZElan 085	Peptide 41.1	H_2N-K(dns)-RQSAGVLGFAPTNIDDTSFHAGCGDTLAIPCRHRSSLISPARPP
ZElan 086	Dynamin 41.2	H_2N-K(dns)-STSTVSTPVPPPVDDTWLQSASSHSPTPQRRPVSSIHPPGRPP
ZElan 120	Dynamin 41.20 (20 mer)	H_2N-K(dns)-STSTVSTPVPPPVDDTWLQS
ZElan 121	Dynamin 41.21 (20 mer)	H_2N-K(dns)-PPVDDTWLQSASSHSPTPQR
ZElan 122	Dynamin 41.22 (20 mer)	H_2N-K(dns)-SHSPTPQRRPVSSIHPPGRPP

Example 5: Binding of Synthetic Peptides and Peptide-Coated Particles to S100 and P100 fraction derived from Caco-2 cells

Caco-2 cell membrane (P100) and cytosolic (S100) fractions were prepared using a modification of the method described in Kinsella, B. T., O'Mahony, D. J. & G. A. Fitzgerald, 1994, J. Biol. Chem. 269(47): 29914-29919. Confluent Caco-2 cell monolayers (grown in 75 cm^2 flasks for up to 1 week at 37°C and 5% CO_2) were washed twice in Dulbecco's PBS (DPBS) and the cells were harvested by centrifugation at 1000 rpm after treatment with 10 mM EDTA-DPBS. The cells were washed 3 times in DPBS and the final cell pellet was resuspended in 3 volumes of ice cold HED buffer (20 mM HEPES (pH 7.67), 1 mM EGTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride (PMSF)). The cells were allowed to swell for 5 min on ice prior to homogenisation for 30 sec. The homogenates were centrifuged at 40,000 rpm for 45 min at 4°C. The supernatant (S100) was removed and the pellet (P100) was resuspended in HEDG buffer (20 mM HEPES (pH 7.67), 1 mM EGTA, 0.5 mM dithiothreitol, 100 mM NaCl, 10% glycerol, 1 mM PMSF). Protein concentrations were determined using the Bradford assay (Bradford, M. M., 1976, Anal. Biochem. 72: 248-254).

Binding of peptide and/or peptide-coated PLGA particles to membrane (P100) and cytosolic (S100) fractions was assessed by detection of the dansyl moiety incorporated in the peptide. Costar ninety six well ELISA plates were coated with S100 and P100 fractions (100 μg/ml in 0.05 M $NaHCO_3$) overnight at 4°C. The plates were blocked with 0.5% bovine serum albumin in DPBS for 1 h at room temperature and washed 3 times in 1% BSA-DPBS. Peptide-coated particles or peptides were dispersed in the same buffer and added to the plates at concentrations in the range 0.0325 - 0.5 mg/well. After 1 h at room temperature the plates were washed 5 times in 1% BSA-DPBS and 100 μl of anti-dansyl antibody (Cytogen DB3-226.3; 0.5 μg/ml) was added per well. The plates were incubated for 1 h at room temperature. The wells were washed 3 times in 1% BSA-DPBS and 100 μl of goat anti-mouse IgG:HRP antibody (Southern Biotechnology 1060-05; 1:10,000) was added per well. The plates were incubated for 1 h at room temperature. After washing 3 times in 1% BSA-DPBS 100 μl of TMB substrate (3,3',5',5-tetramethylbenzidine; Microwell Peroxidase Substrate System (Kirkegaard & Perry Laboratories 50-76-00)) was added and the optical density was measured at 650 nm at various time intervals.

Fig. 5 shows the binding of peptide 41.1 and dynamin 41.2 to P100 (panel A) and S100 (panel B) Caco-2 fractions. The subcellular fractions P100 and S100 were used at a concentration of 50 μg/ml and the background has been subtracted. As shown in Fig. 5, the synthetic peptides were evaluated in ELISA assays for binding to membrane P100 and cytosolic S100 fractions isolated from Caco-2 cells. The samples consisted of peptides: (1) scrambled PAX2-coated nanoparticles, (2) HAX42-coated nanoparticles, (3) peptide 41.1-coated particles, and (4) dynamin 41.2-coated particles. HAX42 is the dansylated peptide:

H_2N-K(dns)SDHALGTNLRSDNAKEPGDYNCCGNGNSTGRKVFNRRRPSAIPT-$CONH_2$

which is disclosed in co-pending US patent application ________, filed May 15, 1998, which application is hereby incorporated by reference in its entirety. HAX42 (positive control) is a promising targeting agent that was discovered, in part, by its

ability to interact with the GIT peptide transport associated receptor HPT1. Scrambled PAX2 (negative control) is a randomly chosen dansylated peptide having the following structure:

H_2N-K(dns)GRNHDVVSSNTHKSYRSPRSASYPRLSNDRTDRTEPAPSS-$CONH_2$

The binding of peptide 41.1 to both P100 and S100 Caco-2 fraction is comparable to the binding of GIT targeting peptide, HAX42. Saturation of peptide binding was observed between 2 and 10 µg/ml for peptide 41.1. No binding was observed for the dynamin human homologous peptide 41.2. Binding of scrambled PAX2 negative control peptide was observed at the highest concentration of peptide used (1µg/ml).

To compare the binding of drug-containing particles with and without being coated with peptides, synthetic peptide 41.1 and dynamin 41.2 were coated onto PLGA nanoparticles loaded with insulin and the binding of particles to membrane P100 and cytosolic S100 fractions isolated from Caco-2 cells were analyzed. Fig. 6 shows that at a particle concentration of 62.5 µg/well, test peptide 41.1-coated particles (TEXP2041 and dynamin 41.2-coated particles (TEXP2042) exhibited greater binding to both P100 (panel A) and S100 (panel A) fractions than the scrambled PAX2 coated control particles or particles with no peptide coating (TEXP 939). For particles TEXP2041 there was a concentration effect with increasing P100 and S100 concentration, while there was not such a trend with the test particle TEXP2042.

Example 6: Animal study

An open-loop study in which the test solution was injected directly into the ileum was undertaken. Wistar rats (300-350g) were fasted for 4 hours and anaesthetized by IM administration 15 to 20 minutes prior to administration of the test solution with a solution of ketamine [0.525 ml of ketamine (100 mg/ml) and 0.875 ml of ACP (2mg/ml)]. The rats were then injected with a test solution (injection volume: 1.5ml PBS) intra-duodenally at 2-3 cm below the pyloris. The test solution contained

polylactide-co-glycolide (PLGA) particles manufactured according to the coacervation formulation 1 given above. Insulin (fast-acting bovine; 28.1 iu/mg) was incorporated in the particles at 300iu insulin (~210 mg particles). Blood glucose values for the rats were measured using a Glucometer (Bayer; 0.1 to 33.3 m/mol/L); plasma insulin values were measured using a Phadeseph RIA Kit (Upjohn Pharmacia; 3 to 240 μU/ml-assayed in duplicate). Systemic and portal blood was sampled.

Insulin loaded, peptide coated PLGA particles (TEXP2041 and TEXP2042) have been analyzed *in vivo* in the rat duodenal open loop model and the profiles of insulin delivery to the systemic circulation is reported in Fig. 7. While the profile of insulin concentration for TEXP2042 particles (dynamin 41.2) is higher than uncoated particles (to be noticed that the value at 3hrs need to be adjusted for a sample missing), the insulin detected in rats treated with TEXP2041 particles (peptide 41.1) is unexpectedly low. A possible explanation of such a discrepancy of data obtained *in vivo* compared to data obtained *in vitro*, could be both the higher speed of uptake and transport of active agent delivered by particles coated with peptide 41.1 and the targeting of those particles to brain tissue where they could be retained, thus reducing the plasma insulin concentration for measurement. The fact that peptide 41.1 had been originally selected as being able to enhance the transport of bacteriophage from the intestinal lumen to the brain supports this premise.

The present invention is not to be limited in scope by the specific embodiments described herein. Various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: O'Mahony, Daniel J
Alvarez, Vernon L
Seveso, Michela

(ii) TITLE OF INVENTION: Peptides Which Enhance Transport of an Active Agent Across Tissues and Compositions and Methods of Using the Same

(iii) NUMBER OF SEQUENCES: 16

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(F) ZIP: 30504

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: WO
(B) FILING DATE: 15-MAY-1998
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/857,046
(B) FILING DATE: 15-MAY-1997

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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     (ix) FEATURE:
          (A) NAME/KEY: CDS
          (B) LOCATION: 5..109


     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCG AGG GGG TAC GGC CGG TTG GCG GAG TCG TGT TGT GTG AAC GAT CGT        49
     Arg Gly Tyr Gly Arg Leu Ala Glu Ser Cys Cys Val Asn Asp Arg
       1               5                  10                  15

TGT ATT CGT ACC GTC GGG GGT TGT GGT AAT TCC CCT GCC TCC GAC ATC         97
Cys Ile Arg Thr Val Gly Gly Cys Gly Asn Ser Pro Ala Ser Asp Ile
                20                  25                  30

CTC TCC AAC ACG                                                        109
Leu Ser Asn Thr
            35


(2) INFORMATION FOR SEQ ID NO:2:

       (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 35 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: protein

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Arg Gly Tyr Gly Arg Leu Ala Glu Ser Cys Cys Val Asn Asp Arg Cys
  1               5                  10                  15

Ile Arg Thr Val Gly Gly Cys Gly Asn Ser Pro Ala Ser Asp Ile Leu
             20                  25                  30

Ser Asn Thr
         35


(2) INFORMATION FOR SEQ ID NO:3:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 124 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)


    (ix) FEATURE:
          (A) NAME/KEY: CDS
          (B) LOCATION: 5..118
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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CTCG AGC ACC CCA GGT CGT GGC TCC GGT CGG GAT ACG GGG GCC AAC AAC     49
     Ser Thr Pro Gly Arg Gly Ser Gly Arg Asp Thr Gly Ala Asn Asn
                 40                  45                      50

GCG GCT GAC ACC CCT TAC GCC AAT CCC TCT CAC CGC GAC ACG ATC CTT      97
Ala Ala Asp Thr Pro Tyr Ala Asn Pro Ser His Arg Asp Thr Ile Leu
                55                  60                  65

TCC CTC GAC CCC TCC CTT CTC TCTAGA                                   124
Ser Leu Asp Pro Ser Leu Leu
            70
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Ser Thr Pro Gly Arg Gly Ser Gly Arg Asp Thr Gly Ala Asn Asn Ala
 1               5                  10                  15

Ala Asp Thr Pro Tyr Ala Asn Pro Ser His Arg Asp Thr Ile Leu Ser
            20                  25                  30

Leu Asp Pro Ser Leu Leu
        35
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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 127 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 5..121

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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CTCG AGG CAG CAC CTC GTC GTT CGT GAC TTG CAT GAG CCT CGT TTC CGC     49
     Arg Gln His Leu Val Val Arg Asp Leu His Glu Pro Arg Phe Arg
         40                  45                  50

GAC ACT AAT ACC GGT GTC CAC GCC ACG TTC TCG CCG CCT GTC TCC GTC      97
Asp Thr Asn Thr Gly Val His Ala Thr Phe Ser Pro Pro Val Ser Val
    55                  60                  65
```

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GCT ACC GAC CAC CGC ACC CCG CCC TCTAGA                                  127
Ala Thr Asp His Arg Thr Pro Pro
 70                 75
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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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Arg Gln His Leu Val Val Arg Asp Leu His Glu Pro Arg Phe Arg Asp
 1               5                  10                  15

Thr Asn Thr Gly Val His Ala Thr Phe Ser Pro Pro Val Ser Val Ala
            20                  25                  30

Thr Asp His Arg Thr Pro Pro
        35
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(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 127 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 5..121

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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CTCG AGT TTC AGC AAC CTT ACC GCC GGT GAT GAG GAG GAT GAT CAC TTC        49
     Ser Phe Ser Asn Leu Thr Ala Gly Asp Glu Glu Asp Asp His Phe
      40                  45                  50

TCG GGT GGG CGG TTC AAT CAC GCC AAT CTT ACT AGC CGG TCC CAT AAT         97
Ser Gly Gly Arg Phe Asn His Ala Asn Leu Thr Ser Arg Ser His Asn
 55                  60                  65                  70

CGT GGG CAG CTG GCT AGT TCC GCC TCTAGA                                 127
Arg Gly Gln Leu Ala Ser Ser Ala
                75
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Ser Phe Ser Asn Leu Thr Ala Gly Asp Glu Glu Asp Asp His Phe Ser
  1               5                  10                  15

Gly Gly Arg Phe Asn His Ala Asn Leu Thr Ser Arg Ser His Asn Arg
             20                  25                  30

Gly Gln Leu Ala Ser Ser Ala
         35
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 127 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 5..121

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
CTCG AGG CAG AGT GTC TTG GAC AGC TGG GGG GGT AAG ACG AGT GTT ACG        49
     Arg Gln Ser Val Leu Asp Ser Trp Gly Gly Lys Thr Ser Val Thr
      40                  45                  50

GGG AGC CTG GAG CGC TAT TAC GCC AGC CAC TCT CAC ACT AGT GCC CCC         97
Gly Ser Leu Glu Arg Tyr Tyr Ala Ser His Ser His Thr Ser Ala Pro
 55                  60                  65                  70

ACT CCC CAC TAC GCC TCC CAC TCT TCTAGA                                 127
Thr Pro His Tyr Ala Ser His Ser
                 75
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Arg Gln Ser Val Leu Asp Ser Trp Gly Gly Lys Thr Ser Val Thr Gly
  1               5                  10                  15

Ser Leu Glu Arg Tyr Tyr Ala Ser His Ser His Thr Ser Ala Pro Thr
            20                  25                  30

Pro His Tyr Ala Ser His Ser
        35
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 127 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 5..121

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
CTCG AGA CAG TGG GTG GGT GAC CGG GCG GAT GGG GAG GGG AAC TGG GTT     49
     Arg Gln Trp Val Gly Asp Arg Ala Asp Gly Glu Gly Asn Trp Val
      40                  45                  50

GAC GAG AAG TAT AGT CGG GAC GCC AAT GTC ATT TCG TAC CGG AAG CAC      97
Asp Glu Lys Tyr Ser Arg Asp Ala Asn Val Ile Ser Tyr Arg Lys His
 55                  60                  65                  70

AAC CAT GCG AGC CAG GGC ACC CTC TCTAGA                              127
Asn His Ala Ser Gln Gly Thr Leu
                75
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Arg Gln Trp Val Gly Asp Arg Ala Asp Gly Glu Gly Asn Trp Val Asp
  1               5                  10                  15

Glu Lys Tyr Ser Arg Asp Ala Asn Val Ile Ser Tyr Arg Lys His Asn
            20                  25                  30

His Ala Ser Gln Gly Thr Leu
        35
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 103 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 5..103

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
CTCG AGG GCG AGT GAT TGT GAT GTC GAG TGT AAC CTG CGC TGG GTG GAG        49
     Arg Ala Ser Asp Cys Asp Val Glu Cys Asn Leu Arg Trp Val Glu
      40                  45                  50

GAT GTG GGG GGG GTG TGG TAC GCC AAG ACC GTT TCG CGA ATG CTA AGC         97
Asp Val Gly Gly Val Trp Tyr Ala Lys Thr Val Ser Arg Met Leu Ser
 55                 60                  65                  70

ACG ACG                                                                 103
Thr Thr
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
Arg Ala Ser Asp Cys Asp Val Glu Cys Asn Leu Arg Trp Val Glu Asp
  1               5                  10                  15

Val Gly Gly Val Trp Tyr Ala Lys Thr Val Ser Arg Met Leu Ser Thr
            20                  25                  30

Thr
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 142 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 5..136

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
CTCG AGA CAG TCT GCG GGC GTG TTG GGT TTT GCG CCT ACC AAT ATC GAT        49
     Arg Gln Ser Ala Gly Val Leu Gly Phe Ala Pro Thr Asn Ile Asp
         35                  40                  45

GAC ACT AGC TTT CAT GCG GGT TGT GGT GAC ACA TTG GCG ATT CCG TGC         97
Asp Thr Ser Phe His Ala Gly Cys Gly Asp Thr Leu Ala Ile Pro Cys
     50                  55                  60

CGA CAT CGT TCC TCC CTG ATC AGC CCT GCT CGC CCT CCC TCTAGA             142
Arg His Arg Ser Ser Leu Ile Ser Pro Ala Arg Pro Pro
 65                  70                  75
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
Arg Gln Ser Ala Gly Val Leu Gly Phe Ala Pro Thr Asn Ile Asp Asp
  1               5                  10                  15

Thr Ser Phe His Ala Gly Cys Gly Asp Thr Leu Ala Ile Pro Cys Arg
            20                  25                  30

His Arg Ser Ser Leu Ile Ser Pro Ala Arg Pro Pro
        35                  40
```

What is claimed is:

1. A transport or uptake targeting agent comprising a targeting peptide having the amino acid sequence substantially as set forth in SEQ ID NO: 16 or a fragment, derivative or peptidomimetic thereof.

2. A transport or uptake targeting agent comprising a targeting peptide having the amino acid sequence substantially as set forth in SEQ ID NO: 2 or a fragment, derivative or peptidomimetic thereof.

3. A transport or uptake targeting agent comprising a targeting peptide selected from the group consisting of an amino acid sequence substantially as set forth selected in SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12 and SEQ ID NO:14 or a fragment, derivative or peptidomimetic thereof.

4. A transport or uptake targeting agent comprising peptide 41.1 or a fragment, derivative or peptidomimentic thereof.

5. A transport or uptake targeting agent comprising dynamin 41.2 or a fragment, derivative or peptidomimentic thereof.

6. The targeting agent of Claim 5, wherein the fragment is selected from the group consisting of dynamin 41.20, dynamin 41.21 and dynamin 41.22.

7. A transport or uptake targeting agent comprising a peptide of not more than 50 amino acids in length which permits or facilitates the transport of an active agent through a human or animal tissue or uptake of the active agent into the human or animal tissue, in which the peptide includes, positioned anywhere along its sequence, a contiguous amino acid sequence selected from the group consisting of RQSAGVL, TNIDDT, RQSVLD, RQWVGDRA,

RQSVLDSWGG, RWQVGDRADGE, RQHLVVRDL, VED, GVHA and RYYA and multiple copies thereof or combinations thereof.

8. A transport or uptake targeting agent comprising a peptide of not more than 50 amino acids in length which permits or facilitates the transport of an active agent through a human or animal tissue or uptake of the active agent into the human or animal tissue, in which the peptide includes, positioned anywhere along its sequence, the contiguous amino acid sequence RQSAGVL and the contiguous amino acid squence TNIDDT.

9. A transport or uptake targeting agent comprising a peptide of not more than 50 amino acids in length which permits or facilitates the transport of an active agent through a human or animal tissue or uptake of the active agent into the human or animal tissue, in which the peptide includes, positioned anywhere along its sequence, the contiguous amino acid sequence RQSAGVLGFAPTNIDDTSFHA or multiple copies of the same.

10. A transport or uptake targeting agent comprising a peptide of not more than 50 amino acids in length which permits or facilitates the transport of an active agent through a human or animal tissue or uptake of the active agent into the human or animal tissue, in which the peptide includes, positioned anywhere along its sequence, the contiguous amino acid sequence STSTVSTPVPPPVDDTTWLQSAS or multiple copies of the same.

11. A drug delivery system comprising at least one targeting agent of any of Claims 1 to 10 in combination with an active agent, wherein the targeting agent permits or facilitates the transport of an active agent through a human or animal tissue or uptake of the active agent into the human or animal tissue.

12. The drug delivery system of Claim 11, wherein the active agent comprises a drug loaded nano- or microparticle.

13. The drug delivery system of Claim 12, wherein the targeting agent is adsorbed or coated onto the nano- or microparticle.

14. The drug delivery system of Claim 12, wherein the targeting agent is directly linked or linked via a linking moiety onto the surface of the nano- or microparticle.

15. The drug delivery system of Claim 12, wherein the nano- or microparticle comprises the targeting agent.

16. The drug delivery system of Claim 11, further comprising a specific receptor targeting agent in combination with the transport or uptake targeting agent.

17. The drug delivery system of any of Claims 11 to 16, wherein the active agent is a drug.

18. A composition comprising the transport or uptake targeting agent of any of Claims 1 to 10 and a Therapeutic.

19. A method of delivering an active agent to a human or animal, comprising administering the drug delivery system of any of Claims 11-17 to the human or animal.

20. The method of Claim 19, wherein the administering step is oral.

21. The method of Claim 19, wherein the administereing step is parenteral.

22. The method of Claim 19, wherein the active agent is insulin.

23. The method of Claim 19, wherein the acitve agent is leuprolide or a salt thereof.

24. A method of treating or preventing a disease comprising administer to a subject in which such treatment or prevention is desired a therapeutically effective amount of the drug delivery system of any of Claims 11-17.

25. The drug delivery system of Claim 11 for use as a medicament.

26. A nucleic acid comprising a nucleotide sequence selected from the group consisting of SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13 and 15 or multiple repeats thereof.

27. An antibody to the transport or uptake targeting agent of any of Claims 1-10.

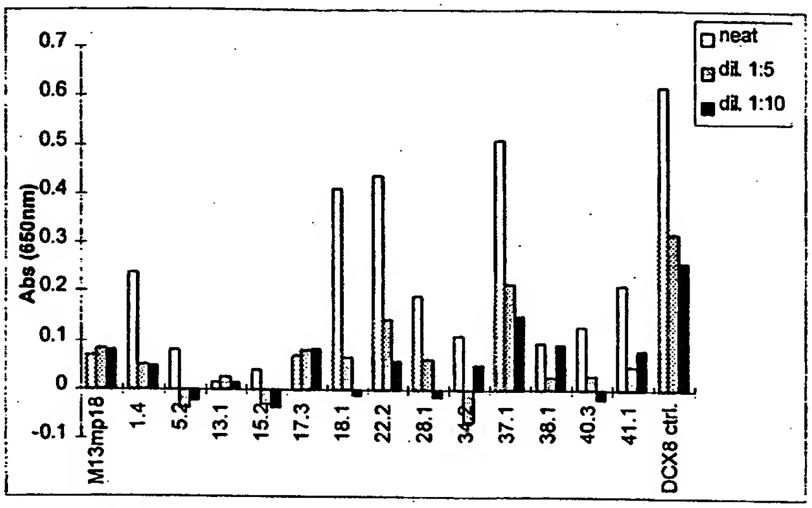


Fig. 1(A)

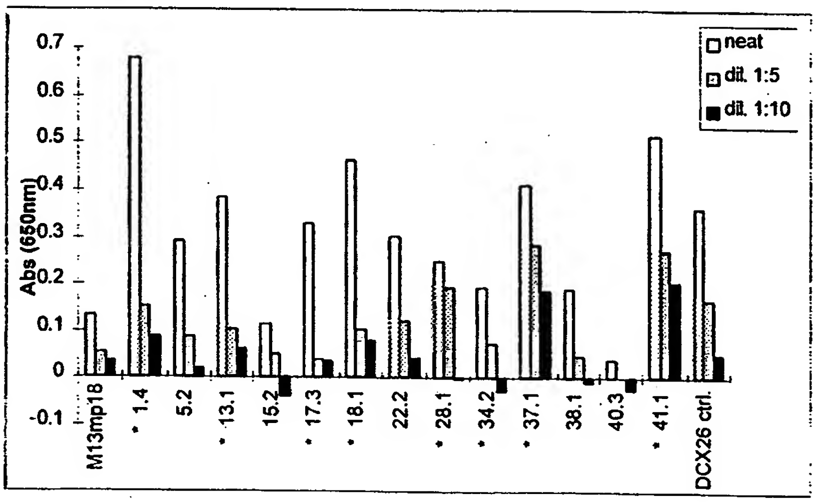


Fig. 1(B)

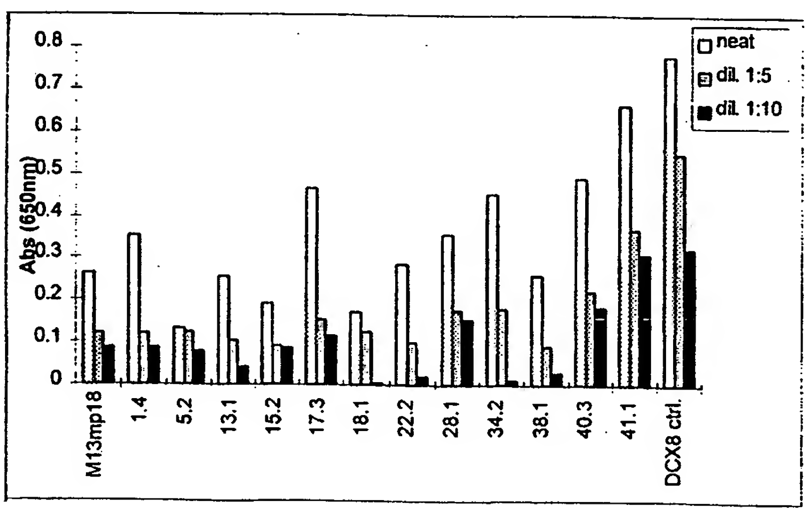


Fig. 1(C)

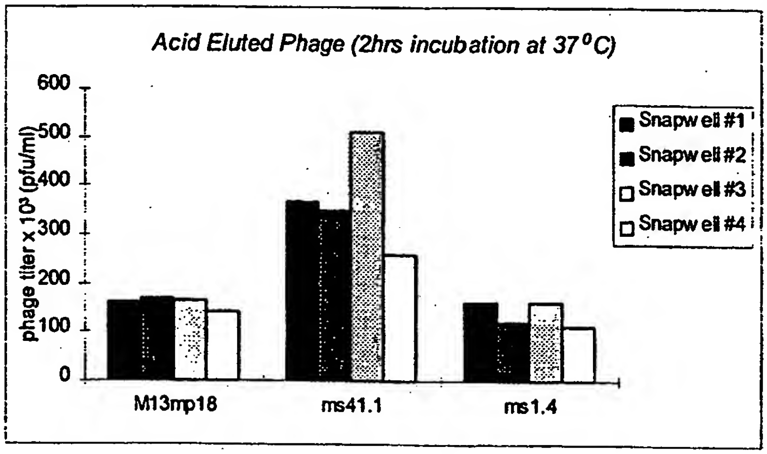


Fig. 2(A)

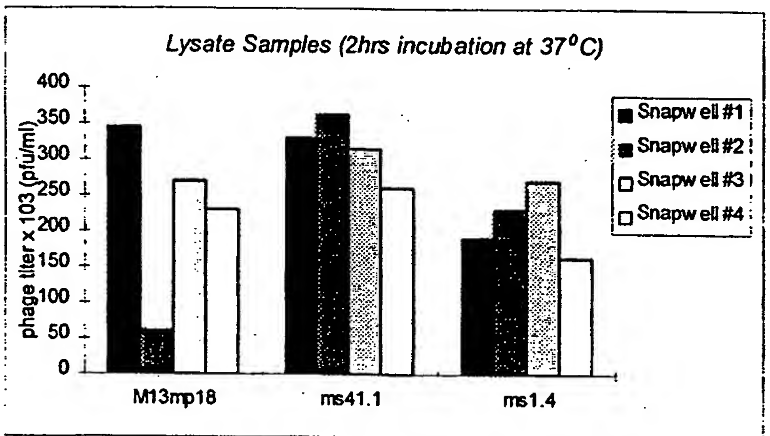


Fig. 2(B)

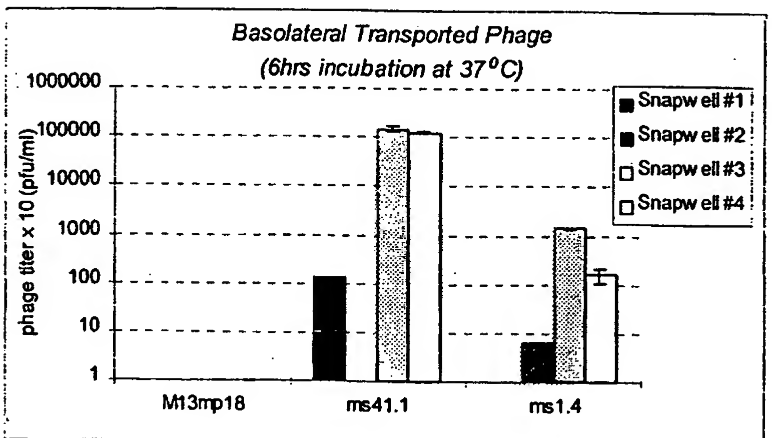


Fig. 2(C)

Sequences producing High-scoring Segment Pairs:	Smallest Sum High Score	Probability P(N)	N
Peptide 1.4			
|P28879| CONST ALPHA-CONOTOXIN SII (S2)	30	0.0075	2
|P35556| HUMAN FIBRILLIN 2 PRECURSOR	41	0.14	3
|P21226| PEA ENDOCHITINASE A2 PRECURSOR	36	0.17	2
|P35555| HUMAN FIBRILLIN 1 PRECURSOR	43	0.18	3
|P98133| BOVIN FIBRILLIN 1 PRECURSOR	41	0.18	3
|P05685| BOMMO CHORION CLASS B PROTEIN B.L1 (410)	38	0.20	2
|P26372| SHEEP KERATIN, ULTRA HIGH-SULFUR MATRIX PR...	38	0.20	2
|P08828| BOMMO CHORION CLASS B PROTEIN L12 PRECURSOR	38	0.22	2
|P02805| SCYSE METALLOTHIONEIN-I (MT-I)	49	0.23	1
|P51004| XENLA POLY(A) POLYMERASE TYPE 1 (PAP) (POL...	40	0.34	2
Peptide 41.1			
|P12823| DEN2P GENOME POLYPROTEIN (CONTAINS: CAPSID...	54	0.039	2
|P39054| MOUSE DYNAMIN 2 (DYNAMIN UDNM)	*43*	*0.067*	*2*
|P50570| HUMAN DYNAMIN 2	*43*	*0.067*	*2*
|P46941| CAEEL HYPOTHETICAL 94.2 KD PROTEIN C38D4.5...	42	0.087	2
|P07720| TBEVS GENOME POLYPROTEIN (CONTAINS: CAPSID...	50	0.095	2
|P06935| WNV GENOME POLYPROTEIN (CONTAINS: CAPSID...	51	0.13	2
|P06022| BPMU POSITIVE REGULATOR OF LATE TRANSCRIP...	34	0.15	2
|P29990| DEN26 GENOME POLYPROTEIN (CONTAINS: CAPSID...	49	0.17	2
|P29991| DEN27 GENOME POLYPROTEIN (CONTAINS: CAPSID...	49	0.17	2
|P07564| DEN2J GENOME POLYPROTEIN (CONTAINS: CAPSID...	49	0.17	2
Peptide 13.1			
|P19419| HUMAN ETS-DOMAIN PROTEIN ELK-1	63	0.016	1
|P41969| MOUSE ETS-DOMAIN PROTEIN ELK-1	54	0.23	1
|P33329| YEAST SERINE/THREONINE PROTEIN PHOSPHATASE...	39	0.29	2
|P17111| DROVI CHORION PROTEIN S36	37	0.43	2
|P36740| HPV40 MAJOR CAPSID PROTEIN L1	34	0.47	3
|Q07875| HPV65 MINOR CAPSID PROTEIN L2	38	0.55	2
|P19274| TTV1 VIRAL PROTEIN TPX	50	0.60	1
|P19275| TTV1V VIRAL PROTEIN TPX	50	0.61	1
|P03211| EBV EBNA-1 NUCLEAR PROTEIN	50	0.62	1
|P39717| YEAST HYPOTHETICAL 95.1 KD PROTEIN IN CNE1...	37	0.63	2
Peptide 17.3			
|P55065| MOUSE PHOSPHOLIPID TRANSFER PROTEIN PRECURSOR	33	0.12	3
|P11979| FELCA PYRUVATE KINASE, M1 (MUSCLE) ISOZYME	43	0.17	2
|P14786| HUMAN PYRUVATE KINASE, M2 ISOZYME	43	0.17	2
|P14618| HUMAN PYRUVATE KINASE, M1 (MUSCLE) ISOZYME...	43	0.17	2
|P35489| ACHLA DIHYDROLIPOAMIDE ACETYLTRANSFERASE C...	36	0.23	2
|P09814| TVMV GENOME POLYPROTEIN (CONTAINS: N-TERM...	43	0.25	2
|P47951| CRIGR SYNDECAN-1 PRECURSOR (SYND1)	51	0.33	1
|P09846| METTH DNA-DIRECTED RNA POLYMERASE SUBUNIT A'	51	0.35	1
|Q01901| PRSVH GENOME POLYPROTEIN (CONTAINS: N-TERM...	41	0.42	3
|P47786| SAGOE NONSECRETORY RIBONUCLEASE PRECURSOR ...	50	0.43	1

Fig. 3(A)

Sequences producing High-scoring Segment Pairs:	Smallest Sum High Score	Probability P(N)	N
Peptide 18.1			
|P32226| SPVKA HYPOTHETICAL PROTEIN C6	63	0.012	1
|P34657| CAEEL HYPOTHETICAL 30.2 KD PROTEIN ZK632.1...	52	0.32	1
|P50350| RHIME PROBABLE TRANSCRIPTIONAL REGULATORY ...	36	0.46	2
|P26444| BOVIN BETA CRYSTALLIN A2	36	0.46	2
|P41884| CAEEL HYPOTHETICAL 43.5 KD PROTEIN F37A4.6...	49	0.66	1
|P23904| BACMA BETA-GLUCANASE PRECURSOR (ENDO-BETA-...	37	0.67	2
|P14448| CHICK FIBRINOGEN ALPHA AND ALPHA-E CHAIN P...	49	0.68	1
|P53214| YEAST HYPOTHETICAL 57.5 KD PROTEIN IN VMA7...	42	0.71	2
|P19402| CAVPO CORTICOTROPIN-LIPOTROPIN PRECURSOR (...	48	0.75	1
|P16070| HUMAN CD44 ANTIGEN, HEMATOPOIETIC FORM PRE...	48	0.76	1
Peptide 28.1			
|P08170| SOYBN SEED LIPOXYGENASE-1 (L-1)	50	0.098	2
|P09439| SOYBN SEED LIPOXYGENASE-2 (L-2)	50	0.13	2
|P80492| BRAFL BRACHYURY PROTEIN HOMOLOG 2 (T PROTEIN)	46	0.20	2
|P16396| BACSU MINOR EXTRACELLULAR PROTEASE EPR PRE...	54	0.21	1
|P02008| HUMAN HEMOGLOBIN ZETA CHAIN	52	0.28	1
|P06347| PANTR HEMOGLOBIN ZETA CHAIN	52	0.28	1
|Q09733| SCHPO HYPOTHETICAL 126.5 KD PROTEIN C31A2....	53	0.29	1
|P27765| PSECL AMIDASE	37	0.30	2
|P43926| HAEIN ELONGATION FACTOR TU (EF-TU)	37	0.30	2
|Q03603| CAEEL PROBABLE DIACYLGLYCEROL KINASE (DIGL...	47	0.35	2
Peptide 34.2			
|Q10310| SCHPO HYPOTHETICAL 59.0 KD PROTEIN C6C3.06...	44	0.17	2
|Q04575| SHVX RNA REPLICATION PROTEIN (CONTAINS: R...	53	0.27	1
|P20485| YEAST CHOLINE KINASE	44	0.32	2
| | [Segment 2 of 2] NITROGENASE MOLYBDE...	51	0.41	1
|P30740| HUMAN LEUKOCYTE ELASTASE INHIBITOR (LEI) (...	29	0.46	3
|Q05819| FLAHE HEPARIN LYASE I PRECURSOR (HEPARINAS...	38	0.56	2
|P20951| PMV RNA REPLICATION PROTEIN (CONTAINS: R...	42	0.63	2
|P23169| ASFB7 PROTEIN P22 PRECURSOR (PROTEIN K'177)	37	0.64	2
|P34907| DICDI CYCLIC AMP RECEPTOR 2	36	0.66	2
|P16689| ECOLI PHNM PROTEIN	35	0.66	2
Peptide 37.1			
|P38984| CHLVR 40S RIBOSOMAL PROTEIN SA (P40) (33 K...	57	0.051	1
|P00731| RAT CARBOXYPEPTIDASE A1 PRECURSOR	52	0.24	1
|P15085| HUMAN CARBOXYPEPTIDASE A1 PRECURSOR	51	0.32	1
|P49182| MOUSE HEPARIN COFACTOR II PRECURSOR (HC-II)	50	0.41	1
|P55213| RAT APOPAIN PRECURSOR (CYSTEINE PROTEASE...	38	0.47	2
|P19318| ECOLI RESPIRATORY NITRATE REDUCTASE 2 BETA...	31	0.50	3
|P34137| DICDI PROTEIN-TYROSINE PHOSPHATASE 1 (PROT...	48	0.64	1
|P18530| MOUSE IG HEAVY CHAIN PRECURSOR V REGION (7...	47	0.66	1
|Q04205| CHICK TENSIN	48	0.66	1
|P54697| DICDI MYOSIN IJ HEAVY CHAIN	48	0.66	1

Fig. 3(B)

```
clone 41.1                   L  G  F  A  P  T  N  I  D  D  T ---------  R  H  R  S  S  L  I  S  P  A  R  P  P

                             +  G        P     N     +  D
viral protein                I  G  R  N  P  R  N  E  N  D

                                         P        +  D  D  T           R        S  S  +        P     R  P  P
human dynamin           749              P  P  P  V  D  D  T  755 769  R  P  V  S  S  I  H  P  P  G  R  P  P  782

                                         P     N  +  D
human dynamin           176              P  A  N  M  D        180

                                      +  P  T  N  +
YLE5 CAEELprot.                       S  P  T  N  L  R
```

Fig. 4(A)

```
clone 37.1                   A  S  D  C  D  V  E  C  N  L  R     V  E  D  V  G  G  V     Y  A  K  T  V  S  R

                                   D  C     +     C  N     R     +  +     +  G     +  W  +
laminin binding              158   D  C  A  I  P  C  N  N  R  G  I  Q  S  I  G  T  M  W  W  176
protein

                                                              W  V              G  V  W  +  A  K     +  +  +
rat carboxypeptidase                                     183  W  V  T  Q  A  S  G  V  W  F  A  K  K  I  T  K  198
precursor

                                                              W  V              G  V  W  +  A  K     +  +  +
human                                                    183  W  V  T  Q  A  S  G  V  W  F  A  K  K  I  T  Q  198
carboxypeptidase
precursor

                             A  +  D     +  +  +  C  +  +        +  E     V  G  G  +
mouse heparin           314  A  N  D  Q  E  L  D  C  D  I  L  Q  L  E  Y  V  G  G  I  332
cofactor precursor
```

Fig. 4(B)

clone 1.4		**R**	**L**	**A**	**E**	**S**	**C**	**C**	**V**	**N**	**D**	**R**	**C**	**I**	**R**	**T**	**V**	**G**	
							C	C		N			C						
Alpha-conotoxin						2	C	C	C	N	P	A	C	8					
		+	+			+		C		N		+	C	I		T	+	G	
human fibrillin 2 precursor	2453	K	V	M	P	N	L	C	T	N	G	Q	C	I	N	T	M	G	2469
								C	+	N		R	C	I		T	V		
human fibrillin 2 precursor	504							C	L	N	G	R	C	I	P	T	V		513
			L	+	+	+		C		N		+	C	+			+	G	
human fibrillin 2 precursor	1205		L	S	D	N	L	C	R	N	G	K	C	V	N	M	I	G	1220
								C		N		R	C	+			+	G	
human fibrillin 2 precursor	2348							C	E	N	G	R	C	V	N	I	I	G	2358
								C	V	N			C	+		T		G	
human fibrillin 2 precursor	1541							C	V	N	G	L	C	V	N	T	P	G	1551
							C		+	N		+	C	+		T		G	
human fibrillin 2 precursor	1418						C	S	I	N	A	Q	C	V	N	T	P	G	1429

Fig. 4(C)

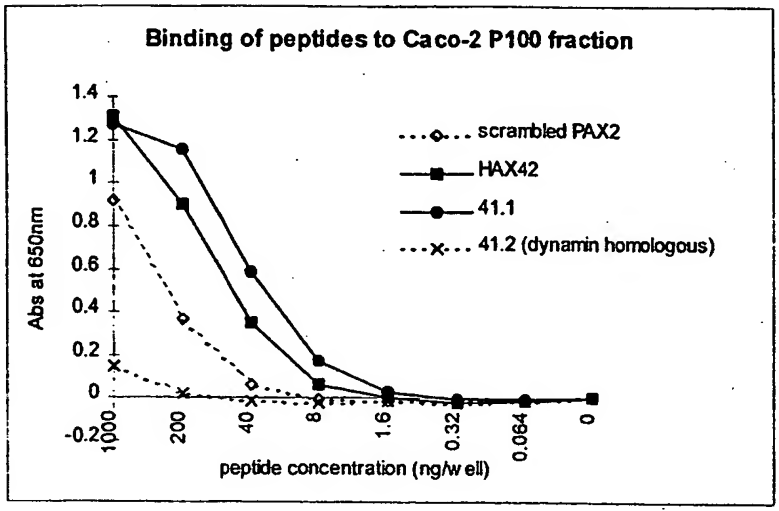

Binding of peptides to Caco-2 P100 fraction
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
Abs at 650nm
scrambled PAX2
HAX42
41.1
41.2 (dynamin homologous)
1000
200
40
8
1.6
0.32
0.064
0
peptide concentration (ng/w ell)

Fig. 5(A)

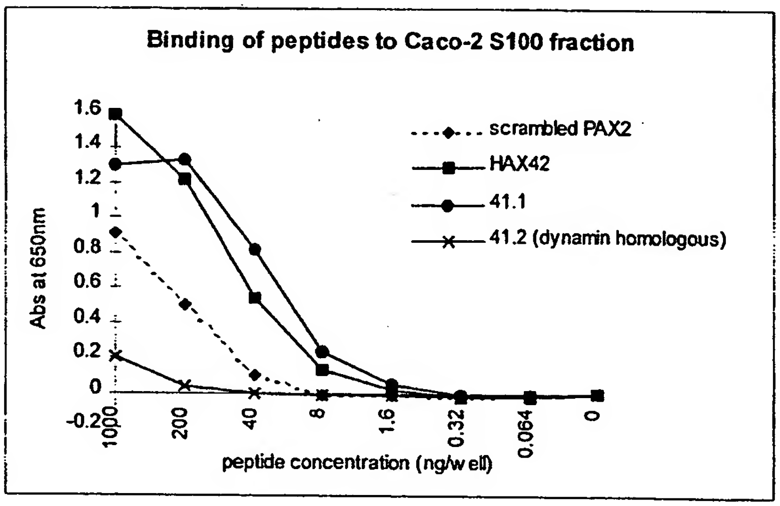
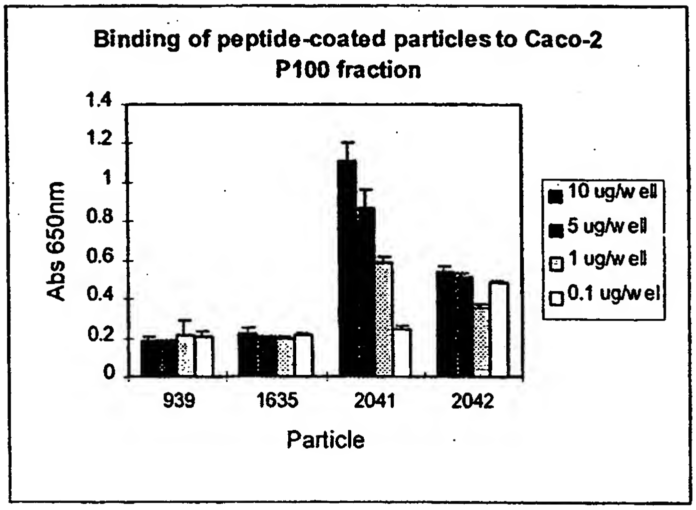
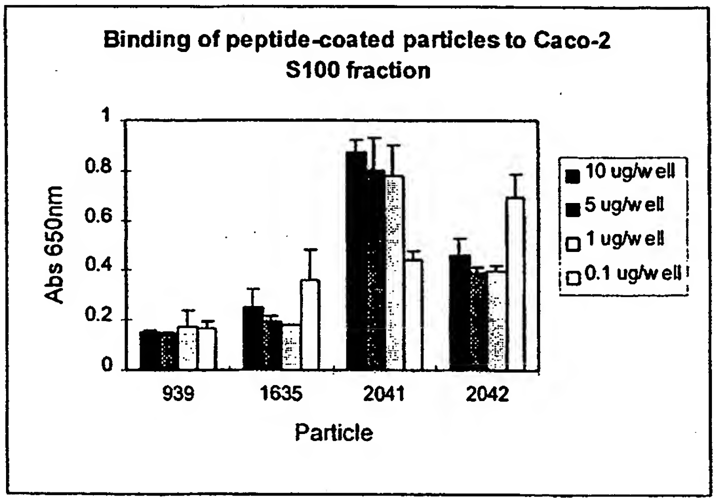

Binding of peptides to Caco-2 S100 fraction
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
Abs at 650nm
scrambled PAX2
HAX42
41.1
41.2 (dynamin homologous)
1000
200
40
8
1.6
0.32
0.064
0
peptide concentration (ng/w ell)

Fig. 5(B)



Binding of peptide-coated particles to Caco-2
P100 fraction
Abs 650nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
939
1635
2041
2042
Particle
10 ug/well
5 ug/well
1 ug/well
0.1 ug/wel

Fig. 6(A)



Binding of peptide-coated particles to Caco-2
S100 fraction
Abs 650nm
1
0.8
0.6
0.4
0.2
0
939
1635
2041
2042
Particle
10 ug/well
5 ug/well
1 ug/well
0.1 ug/well

Fig. 6(B)

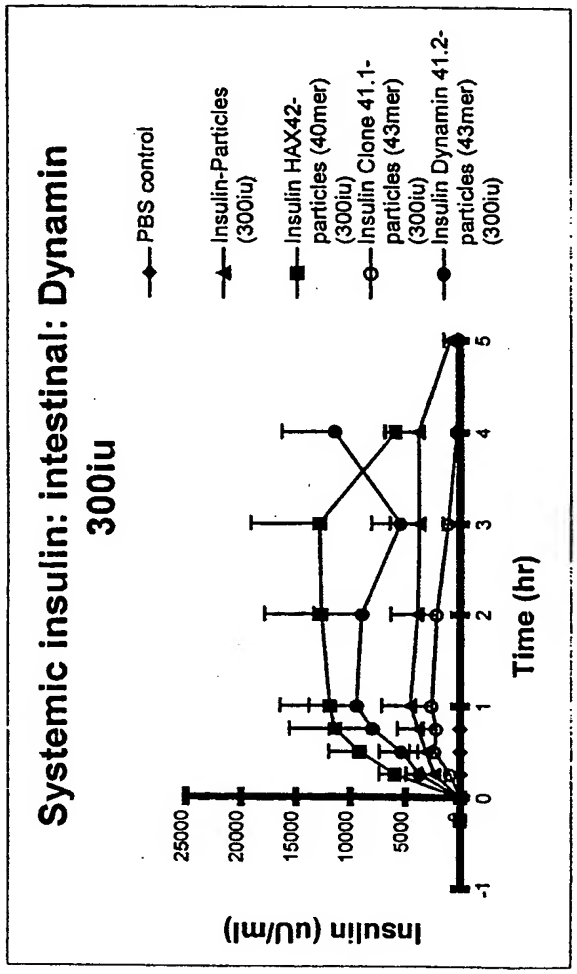

Systemic insulin: intestinal: Dynamin
300iu
Insulin (uU/ml)
25000
20000
15000
10000
5000
0
-1
0
1
2
3
4
5
Time (hr)
PBS control
Insulin-Particles (300iu)
Insulin HAX42-particles (40mer) (300iu)
Insulin Clone 41.1-particles (43mer) (300iu)
Insulin Dynamin 41.2-particles (43mer) (300iu)

Fig. 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/10079

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; C07K 5/00
US CL :435/6; 530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,902,505 A (PARDRIDGE et al.) 20 February 1990, see entire document.	1-16, 25-27
Y	US 5,574,142 A (MEYER, Jr. et al.) 12 November 1996, see entire document.	1-16, 25-27
Y	US 5,589,392 A (SHORT) 31 December 1996, see entire document.	1-16, 25-27
Y,P	US 5,689,039 A (BECKER et al.) 18 November 1997, see entire document.	1-16, 25-27

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 AUGUST 1998	03 SEP 1998

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	EGGERTON CAMPBELL
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/10079

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 17-24
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.